PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

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STUDIES IN ANIMAL AGGREGATIONS: FURTHER ANALYSIS OF THE PROTECTIVE VALUE OF BIOLOGICALLY CONDITIONED FRESH WATER FOR THE MARINE TURBELLARIAN, PROCERODES

W. C. ALLEE
Marine Biological Laboratory and University of Chicago

THE MARINE turbellarian flatworm, *Procerodes wheatlandi*, lives in the intertidal and adtidal zones in restricted regions along the coast of New England. Previous work has shown that they will survive longer in fresh water, other conditions being equal, (*a*) if they are present in numbers; (*b*) if isolated into fresh water in which other living *Procerodes* have previously been exposed and especially (*c*) if isolated into fresh water in which some few *Procerodes* have recently died and disintegrated, even though such a conditioned medium be boiled and filtered; and (*d*) if the salinity of the fresh water is increased by adding as much as 0.25 per cent of sea water above a minimum of that value. The protective material will stand boiling and will pass through filter paper (Allee, 1928).

The protective value of this biologically conditioned fresh water is maintained after dialysis to the same electrical resistivity as that of extremely dilute sea-water controls. Water extracts of marine amphipods and of the fresh-water planarian, *Planaria maculata*, show the same effects as does also water from cultures of the latter and heated hay infusions from a practically pure culture of *Paramecium*. The protective effects are not due to difference in pH or to

depression such as is produced by exposure to dilute alcohol; gelatine suspensions do not have similar protective value (Allee, 1929).

There were some indications found near the close of this earlier work that adsorption on charcoal lessened the protective value of *Paramecium* culture medium, but otherwise there were no clues to the nature of the protective mechanism. At the first opportunity experimentation was resumed, using the technique previously developed and described in the 1929 report. The experiments were again carried on at the Marine Biological Laboratory at Woods Hole.

In preceding studies fresh *Planaria maculata* culture water had shown high protective value, as compared with extremely hypotonic sea water of equal electrical resistance.[1] For the first part of the experiments to be reported here, attention was focused on this medium. The *Procerodes* were collected from the same locality as before and given similar treatment before experimentation. Worms of the same laboratory age were selected for any given experiment. They were washed through five changes of fresh water and then isolated into chemically clean watch glasses of the egg-cup type; excess liquid was removed; and the isolated worms were immersed in 1 ml. of the appropriate test or control solution. The tests were made normally with sets of ten worms for each aspect of the experiment; frequently, more than one set of ten worms was exposed to the same conditions at the same time. Within any one experiment, all the worms in the same ordinal rank were selected to be as nearly alike in size and activity as was possible by mere observation.

After isolation, the various watch glasses were placed in moist chambers and left at room temperature. The temperature was approximately uniform for a given experiment, but there was some effect of the usual daily range in the longer ones. The laboratory room had a north exposure only, and room temperatures during the experimental season varied between 22° and 26.5° C.

The worms were inspected with an 8-power hand lens immediately after the experiment was started and at appropriate intervals until all had begun to cytolyze. Early in an experiment these intervals

[1] A different set-up was used for measuring conductivity from that of the preceding tests; with the apparatus used, a N/10 solution of KCl by weight only at 23.75° C. showed a resistance of 26 ohms.

were about 30 minutes; later, when changes were slowed down, inspections were made every hour; and still later, particularly in the experiments that ran past midnight, inspections were made at intervals of 2 hours. In the last experiment of the season, No. 99, worms surviving overnight were placed in a cold chamber to retard cytolysis during the early morning hours. This experiment is excluded from all discussions unless specifically mentioned.

The rate of onset of cytolysis in hypotonic waters depends on a number of factors, among which are the following: Small worms begin and undergo cytolysis more rapidly than do larger ones. Worms long in the laboratory are less resistant than are those freshly collected. The higher the temperature, the lower the concentration of the sea water; and the larger the proportion of distilled water, as contrasted with tap or pond water, the sooner cytolysis begins. Accordingly, in any given experiment all these factors were kept as constant as possible.

Worms may recover after cytolysis begins without transfer to sea water if the area affected is small, if the worms are in good condition, and if the water to which they are exposed is too not strongly lethal. Under the conditions of these experiments, such recoveries did not usually occur. When they did take place, survival time is taken as the time at which cytolysis leading to death begins. In all cases in this report, therefore, "survival time" really means the length of survival before the onset of fatal cytolysis could be detected. The physiological and histological problems involved in cytolysis are of interest, and some attention has been paid to certain aspects of these problems, but their consideration is deferred for the present.

Preceding work on the survival time of *Procerodes* isolated into different kinds of extremely hypotonic waters had been performed in September or later in the autumn; accordingly, it was necessary to test whether the same kind of protective effects could be found during the summer. Later this series of comparisons was extended in the controls which accompanied other lines of experimentation until, in all, survival records were obtained for thirty-four sets of paired experiments, which usually had ten pairs of worms in each set. Table I gives a summary of these tests, which were made between July 9 and August 28, 1931.

The mean of all these tests shows that the worms survived, on the average, 4.44 hours in the planarian culture water, while their accompanying pairs lived 3.13 hours under similar conditions in control waters in which the electrical resistance had been brought up

TABLE I

COMPARATIVE SURVIVAL TIME OF *Procerodes* ISOLATED INTO *Planaria maculata* CULTURE MEDIA AND INTO HYPOTONIC SEA WATER OF THE SAME ELECTRICAL RESISTANCE

Exp. No.	*Planaria maculata* Culture Water			Control			Temp. (Degrees Cent.)
	Resistance in Ohms	No. of Worms	Hours of Survival	Resistance in Ohms	No. of Worms	Hours of Survival	
75[a]	3,560	10	3.62	3,440	10	2.48	24
	4,060	10	3.36	4,060	10	1.86	24
76	11,000	10	1.77	7,000	10	1.71	24
	11,000	10	3.23	7,000	10	2.68	24
77	10,000	10	1.68	10,000	10	1.46	25
	10,000	10	2.04	10,000	10	1.60	25
78	4,000	10	9.53	4,000	10	9.48	24
	4,000	10	8.66	4,000	10	9.18	24
79	5,500	10	2.91	5,500	10	2.31	25
	5,500	10	2.60	5,500	10	2.22	25
80	9,000	12	1.88	9,000	13	1.92	25
	9,000	13	2.47	9,000	13	2.46	25
81	2,300	10	11.93	2,300	10	1.54	24
82	3,100	8	1.06	3,300	8	1.38	24
	3,100	8	1.13	3,300	8	0.94	24
83	7,000	10	1.64	7,000	10	1.23	25.5
	7,000	10	2.37	7,000	10	1.75	25.5
84	6,000*	10	2.38	6,000	10	1.82	22
	6,000†	10	11.36	6,000	10	7.97	22
	6,000*	10	2.38	6,000	20	2.00	22
	6,000†	10	11.36	6,000	20	4.26	22
85	3,800	10	3.80	3,750	10	3.53	24.5
	3,800	10	5.60	3,750	10	2.88	24.5
	3,800	10	6.01	3,750	10	3.53	24.5
86	2,600	9	3.90	2,500	9	7.90	25
	2,600	9	5.50	2,500	9	1.73	25
	2,600	9	9.06	2,500	8	2.88	25
88	5,250	10	3.26	5,000	10	2.55	26.5
89	6,000	10	3.37	6,000	10	2.53	26.5
	6,000	10	3.78	6,000	10	1.93	26.5
90	2,700	8	4.60	2,700	10	3.73	24
91	5,500	9	6.87	5,500	10	3.46	24
96	2,500	10	5.24	2,500	10	3.68	25
	2,500	10	5.05	2,500	10	3.71	
Mean	.....	.....	4.44	.....	.....	3.13	.....

* and * Same data. † and † Same data.

to that of the biologically conditioned water by the dialysis of the latter and by the addition of a few drops of 1 per cent sea water to the former. The difference, 1.31 hours, has a statistical significance, when examined by Student's method for paired experiments, of 0.00096, which means that there are 96 chances in 100,000 of random sampling yielding as great a difference. The resistance of the biologically conditioned medium at the beginning of the experiments ranged from 2,300 to 11,000 ohms; for the controls, from 2,300 to 10,000 ohms. In any given experiment, if the initial resistance of the water was not balanced within the range of experimental error, there were slightly more electrolytes in the control than in the culture water.

The results from similar experiments performed in the autumn of 1928 have been reported previously (Allee, 1929, p. 368). In the earlier series the initial resistance of the tested media ranged from 1,550 to 2,400 ohms, as compared with 2,300 to 11,000 in the series here reported. In the nineteen sets of experiments with 386 worms, the *Procerodes* isolated into *Planaria maculata* culture water lived on the average 48.31 hours, while the accompanying controls in hypotonic sea water lived 25.43 hours; the difference of 22.86 hours has a statistical probability of 0.0002. This long resistance to hypotonic waters is to be compared with the much shorter survival shown in Table I.

Four factors are known to have differed between these two sets of experiments: The earlier ones had a higher concentration of electrolytes. The temperatures were lower. Tap or pond water was the standard fresh water used, while in the later experiments distilled water was frequently used either entirely or mixed with pond water of low conductivity. All these differences would make for longer survival in the earlier series. The fourth variable, the difference in season, cannot be evaluated but may have played an important rôle. The shorter survival in 1931 favored a more exact determination of the time of onset of cytolysis; this, combined with the greater number of experiments, allows the relatively small difference in survival in this set of experiments to approximate the statistical significance of the former.

ADSORPTION EXPERIMENTS

The experiments reported previously (Allee, 1929) yielded a small amount of data which indicated that adsorption on charcoal lessened the protective value of *Paramecium* culture medium for *Procerodes* isolated therein. The data then available are summarized in Table II.

As was concluded at the time, there are indications in these data that charcoal does remove some of the protective material; however, the experiments to test this point were not fully controlled, in that simultaneous tests with adsorbed and unadsorbed culture media

TABLE II

INDICATIONS FROM EARLIER EXPERIMENTS OF ADSORPTION OF PROTECTIVE MATERIAL ON CHARCOAL

Exp. No.	Conditions	Resistance in Ohms		No. of Worms	Hours of Survival			Statistical Probability
		Exp.	Control		Exp.	Control	Difference	
74	Unadsorbed	7,400	7,400	50	25.85	15.20	10.65	0.00002
75	Adsorbed	6,050	6,050	50	11.93	9.21	2.72	.056
74	Unadsorbed	7,400	6,150	50	25.85	19.30	6.55	.02
75	Adsorbed	6,050	5,050	50	11.03	8.50	2.53	0.072

were not made, and as will be seen later, in experiments with as many variables as are present here, indications from comparable but not simultaneous tests are untrustworthy.

The results of adequately controlled experiments upon the effect of adsorption on charcoal are summarized in Table III, which shows the results of paired experiments in which *Planaria maculata* culture medium was divided, half to be used as a control, and the other half to be shaken and then filtered twice through Kahlbaum's animal charcoal and then dialyzed to the same resistivity as found in the unadsorbed fraction.

In twelve tests, using 121 pairs of worms, the difference in survival before the onset of cytolysis was about 15 minutes and is without significance; hence, we must conclude that charcoal did not adsorb protective material from the *Planaria maculata* culture water.

Seven similar adsorption experiments using as the adsorbing

agent egg albumen, which carries the opposite electrical charge similarly showed no significant decrease in survival following the adsorption. In these tests 66 pairs of worms were used; those in the unadsorbed culture water survived 7.59 hours; those in water with the same initial electrical resistance survived 5.22 hours. The difference, 2.37 hours, has a statistical probability of 0.374 and is not significant.

TABLE III

EFFECT OF ADSORPTION ON CHAROCAL ON THE PROTECTIVE VALUE OF *Planaria maculata* CULTURE WATER FOR *Procerodes*

Exp. No.	Unadsorbed			Adsorbed			Temp. (Degrees Cent.)
	Resistance in Ohms	No. of Worms	Hours of Survival	Resistance in Ohms	No. of Worms	Hours of Survival	
75	3,560	10	3.62	5,580	9	4.49	24
	3,560	10	3.36	5,580	10	3.33	24
76	11,000	10	1.77	8,000	10	3.60	24
	11,000	10	3.23	8,000	10	2.68	24
77	10,000	10	1.68	10,000	10	1.50	25
	10,000	10	2.04	10,000	10	1.93	25
78*	4,000	10	9.53	4,000	10	9.06	24
	4,000	10	8.66	4,000	10	8.31	24
79	5,500	10	2.91	5,500	10	3.40	25
	5,500	10	2.60	5,500	10	3.51	25
80	9,000	12	1.88	9,000	12	2.54	25
	9,000	12	3.47	9,000	12	2.58	25
Mean	.....	.....	3.65	.....	.....	3.91	.....

* Dialyzed in pond water; remainder dialyzed in distilled water.

As a further check on the effect of egg albumen and as a further test of the result of the addition of a protein to the experimental medium, six sets of tests were made in which the survival of worms in planarian culture water which had been adsorbed on egg albumen was compared with that given in a 1 per cent suspension of the same albumen. As always, the two sets of media were brought to the same initial electrical conductivity. Under these conditions, of 55 pairs of worms, those in the egg albumen suspension lived 1.66 hours before cytolysis began, while those in the adsorbed planarian culture water lived 5.09 hours. The difference, 3.43 hours, has a statistical probability of 0.0001 when considered as 55 pairs, or of 0.08 when calculated as the mean difference of six sets of 8, 9, or 10 worms. The former figure indicates a significant difference which further experi-

mentation would probably reveal in the other type of calculation. The *Procerodes* isolated into these egg-albumen suspensions showed practically the same survival as did the hypotonic sea-water control of the same initial conductivity. These results support the experiments reported in 1929, which showed that gelatine suspensions in extremely hypotonic sea water did not have protective value for *Procerodes*.

GUM ARABIC

Experiments with dilute solutions (approximately 1 per cent) of gum arabic demonstrated that this soluble polysaccharide with high molecular weight did not protect *Procerodes* from the ill effects of isolation into hypotonic sea water. The solutions tested ranged from 2,600 to 4,000 ohms resistance; six lots of worms exposed in 1 ml. each survived without cytolysis for 3.9 hours, while the accompanying controls which were similar but lacked the gum arabic survived 3.36 hours. The difference is far from being statistically significant; the probability of random sampling giving similar results in 0.73 when a value of 0.01 or less is a good index of significance.

Even the slight osmotic pressure of these solutions, which showed a Δ F. of 0.005° C., or about 45.8 mm. mercury of osmotic pressure, was not large enough to affect the survival of these worms. Experiments on the protective value of osmotic pressure will be reported in the section on results with cane sugar.

ETHYL URETHANE

Stale culture water from fresh-water planarian worms has long been known to have depressing effects upon other similar planarians (Child, 1915). Although previous experiments (Allee, 1929) with dilute ethyl alcohol had shown no survival value from the treatment with this depressant, the effect of another depressing agent, ethyl urethane, was tested, again with negative results. Thirty pairs of worms similarly treated, except for the presence of 0.2–0.1 per cent of ethyl urethane, showed a mean of only 5 minutes difference in the onset of cytolysis from their accompanying controls. Again we are led to the conclusion that the protective value of biologically conditioned fresh water for these marine *Procerodes* does not lie in its possible action as a depressant.

FRACTIONS OF WATER EXTRACTS OF PROCERODES

Other conditions being equal, water extracts of *Procerodes* have been shown to have definite survival value for other *Procerodes* isolated therein (Allee, 1928, 1929). In the experiments reported in 1929, 320 comparable worms tested in nine experimental lots lived 18.58 hours longer in homotypically conditioned fresh water than did their pairs isolated into dilute sea water of the same conductivity. Despite the great variability in experimental procedure during these experiments, the statistical probability was 0.03. Nine more such tests with 84 pairs of *Procerodes* were run during the course of the present experimental series. The mean survival in all these cases was 29.14 hours, as compared with 13.33 hours in the accompanying controls. The difference, 15.81 hours, has a statistical probability of 0.0052. This serves as a firm basis for departure in attempting to find whether one fraction of the homotypically conditioned medium has more protective value than another, or than the whole.

The procedure was as follows: About 175 *Procerodes* carefully washed in five changes of redistilled water were brought to a moist dryness and covered with 20 ml. of ether and allowed to stand overnight. After a few hours in the ether, they were broken up with a glass rod. The next day the whole was filtered through a carefully washed filter paper. The filtrate was evaporated just to dryness on a water bath and dissolved in 30 ml. of pond and distilled water made up 1:1. This contained the portion of the ether-soluble extract that was also water soluble. It showed an electrical resistance of 7,500 ohms, which was reduced to 3,500 by the addition of dilute sea water.

The fragments of *Procerodes* on the filter paper were allowed to dry and were then washed off with a similar mixture of distilled and pond water and brought to the same conductivity with dilute sea water. Seven lots of worms, totaling 67 individuals, survived on the average 9.32 hours in whole *Procerodes* extract of the same conductivity as these fractions; their pairs in the water-soluble ether extract survived only 3.46 hours. The difference, 5.86 hours, has a statistical probability of 0.00006. The 40 worms in four lots in the ether-insoluble fraction showed a mean survival of 3.32 hours, as

compared with one of 5.30 hours in the comparable ether-soluble fraction. This difference, 1.98 hours, has a statistical probability of 0.0058. The details are set forth in Table IV.

Another ether fraction was prepared by extracting with ether a water extract which included the broken body fragments of *Procerodes*. The ether was then removed with a separatory funnel. The ether fraction was treated as above but appeared to have even less protective value than when prepared in the way first described (see Exp. 95, Table IV). Thus the ether-insoluble fraction has definitely

TABLE IV

COMPARATIVE SURVIVAL VALUE OF WHOLE *Procerodes* EXTRACT AND OF CERTAIN FRACTIONS

Exp. No.	Resistance in Ohms	Whole Extract		Ether-Soluble Fraction		Ether-Insoluble Fraction		Temp. (Degrees Cent.)
		No. of Worms	Hours of Survival	No. of Worms	Hours of Survival	No. of Worms	Hours of Survival	
95	1,700	9	6.81	9	0.77	.....	.....	25
		9	6.94	9	1.12	.....	.....	
		9	7.65	9	1.29	.....	.....	
97	3,500	10	9.06	10	5.53	10	3.81	23.5
		10	11.72	10	7.00	10	4.35	
98	3,300	10	11.29	10	4.80	10	2.59	21.5
		10	11.77	10	3.69	10	2.54	

less protective value than has the ether-soluble fraction; and both, as prepared, are definitely less protective than is the whole, untreated watery extract.

The situation as regards protection from hypotonic water is shown by these tests to be different from that reported by Peebles (1929) for the effects of extracts of plutei upon the rate of cleavage and upon the rate of growth in *Arbacia* eggs and plutei. She found both growth-inhibiting and growth-promoting substances present in such extracts; the inhibiting substances are associated with the lipoid constituents, while the accelerating factor is contained in the protein molecule.

CANE-SUGAR SOLUTIONS

This is not the place to review the whole problem of the use of cane sugar or other non-electrolytes to secure osmotic effects com-

parable with those produced by solutions of electrolytes. It has been the general experience that marine animals transferred to a solution of non-electrolytes isotonic with sea water lose salts from the blood and that, if soft-skinned, they shrink. Cane sugar is not toxic per se, although transfer into isotonic cane-sugar solutions frequently results in death by a loss of salts from the body liquids and their consequent viscous condition. If transferred back into sea water, such animals swell, since they take up water from the less-concentrated normal sea water; later they also take up salts and again come into water balance with their environment (cf. Bethe, 1930).

Sea water has an osmotic pressure of approximately 22.5 atmospheres. Accordingly, a gram-molecular solution of crystallized cane sugar (Baker's analyzed was used throughout) was made in laboratory distilled water. The electrical conductivity was about that of the distilled water. The last of eight *Procerodes*, isolated, as usual, into a distilled-water control, had started toward fatal cytolysis within 2.17 hours, with a mean time of 1.76 hours for all before cytolysis started. When the last of the control worms had begun to disintegrate, the 10 worms in M/1 cane sugar were all alive and intact. They had lost in volume and were more deeply brown in color, and were wrinkled in appearance in place of being velvety smooth. Some tended to round up, and superficially resembled somewhat a wizened precytolysis stage of a much smaller worm in extremely hypotonic sea water. One of these was transferred to full-strength sea water 30 minutes after the last control had begun to disintegrate, and within 5 minutes it had recovered and was crawling normally. The approximate time of death of these worms was not determined.

In M/2 cane-sugar solution without the addition of any electrolytes, the experimental worms lived at least 8.5 hours, as shown by their recovery on transfer to sea water; while the accompanying set of controls in dilute sea-water with a resistance of 5,000 ohms survived 2.55 hours.

The picture presented by these worms in the stronger solutions of cane sugar is totally different from that in hypotonic sea water, even though the latter contain a much higher concentration of electrolytes. In these solutions, cytolysis did not take place, and the only way available for determining the death-point was by transferring back to sea water after different exposure periods.

When a M/6 cane-sugar solution was treated with dilute sea water to bring the solution to 6,000 ohms resistance, the last three *Procerodes* isolated therein were transferred to sea water after 22.5 hours' exposure in the sugar solution, and all survived; the accompanying controls in water of similar conductivity survived, on the average, 2.23 hours, and those lasting longest survived only 3.42 hours. Twelve of 20 *Procerodes* of a different lot recovered completely from an exposure of 21 hours to M/5 cane sugar; while the accompanying controls lasted, on the average, 3.46 hours. The worms in M/5 fared better than did those in M/10, M/15, or M/20, all of which gave a longer survival than did the series of worms tested simultaneously in *Planaria maculata* culture medium, which, as previously shown, has proved to be definitely protective as compared with otherwise similar hypotonic sea-water controls.

Worms isolated into M/40 and M/50 solutions of cane sugar brought to a resistivity of 2,500 ohms by the addition of dilute sea water showed about the same survival as did worms tested simultaneously in similar water that lacked the cane sugar. The mean survival time of 40 worms in the sugar solutions was 3.53 hours; of 20 in the controls, 3.70 hours.

Twenty *Procerodes* isolated into M/$33\frac{1}{3}$ cane-sugar solution made to 2,700 ohms resistance with dilute sea water survived, on the average, 9.67 hours. The accompanying hypotonic sea-water controls survived 3.06 hours less; the random sampling probability is 0.0038. The worms in this cane-sugar solution survived 5.53 hours shorter time than did accompanying similar ones exposed to *Planaria maculata* culture water. This latter difference had a random sampling probability of 0.128, which is much too great to have statistical significance. These experiments are shown in detail in Table V.

Another set of similar experiments in which the *Procerodes* were tested in the planarian culture medium and in M/25 cane-sugar solution, as usual with similar initial conductivity, showed a mean survival time of 5.15 hours for those in the planarian culture water, as compared with 7.05 for those in the sugar solutions. The difference of 1.9 hours has a random sampling probability of 0.0000. These experiments indicate that, other conditions being equal, the *Planaria maculata* culture water has a protective value approximate-

ly equal to a cane-sugar solution of a strength between M/25 and M/$33\frac{1}{3}$. This is equivalent to an osmotic pressure of from 0.45 to 0.56 atmospheres.

The cane-sugar solutions owe their protective value to their osmotic properties. Is is possible that the planarian culture water has osmotic properties other than those produced by introduced or uneliminated electrolytes? Evidence was collected on this point as follows.

Planaria maculata culture medium prepared by allowing the planarians to live in pond water with low conductivity showed resistance of 5,000 ohms. Enough dilute sea water was added to distilled water to bring it to the same resistance. Electrolytes at the low concentration found in such solutions are supposedly all ionized, and the osmotic pressures caused by electrolytes would vary with the specific conductance and the type of electrolytes. To guard against such sources of error, a collodion membrane known to be permeable to electrolytes, and slightly, if at all, permeable to the protective material in biologically conditioned solutions, was filled with the planarian culture water and firmly fastened to the end of a long capillary volumetric pipette of 0.2 ml. capacity. The seal between bag and glass was water-tight. The bag was immersed in the very dilute sea water just prepared, and the height of the column was marked. After slight preliminary oscillation of 1.0 mm. or less, the next afternoon, 23 hours after starting, the osmometer stood 1 mm. below the starting-point. The experiment shows that with electrolytes balanced, there is no osmotic pressure exerted by the *P. maculata* culture medium; hence, its protective value must lie elsewhere.

The same conclusion was reached by using the freezing-point method. These tests were made at the University of Chicago, using Hull Zoölogical Laboratory well water and *Planaria dorotocephala* as conditioning agents. The results of this analysis are listed in Table V.[2]

These data show a slight fixation of electrolytes and an accompanying slight decrease in the osmotic pressure of the well water as a result of the planarian conditioning. There is no evidence such as

[2] Data by R. Oesting, chemical research assistant.

would aid us in accounting for the protective value of the conditioned medium for the marine *Procerodes* isolated therein. The nature of the protective material and of its action is still unexplained by these experiments; however, the tests with cane-sugar solutions do give a measure in terms of osmotic pressure of the extent of the protection furnished.

TABLE V

EFFECT OF *Planaria dorotocephala* UPON ELECTRICAL CONDUCTANCE AND FREEZING-POINT OF OTHERWISE UNCONDITIONED WELL WATER

SPECIFIC CONDUCTANCE IN MHO.		Δ F.	
Unconditioned	Conditioned	Unconditioned	Conditioned
0.0003151	0.0003037	0.014° C.	0.009
Difference			
+0.0000114	.....	+0.005	.....

COMPARATIVE EXPERIMENTS

The protection afforded by these biologically conditioned hypotonic solutions is not due to the mere presence of simple protein particles; otherwise gelatine or egg albumen suspensions would be protective. It is not due to the presence of a dissolved gum, for tests showed that gum arabic is not effective. One of the materials which planarian worms give off into their culture medium is mucus. On the possibility that this might be a protective agent, the protective effect of desiccated parotid gland was tested. When dissolved in water, this material gives a large amount of mucus. The mucus so produced has been shown to have a protective effect for animals if placed in suspensions of colloidal silver (Allee and Schuett, 1927). The protection from colloidal silver furnished in this way resembles that given by snail mucus or by the presence of many planarians. When appropriately controlled tests of the survival value of water plus desiccated parotid gland were made, the results, like those with gum arabic and with gelatine and egg albumen, were negative.

These facts, along with others, are illustrated in Table VI, which shows in detail the survival of worms in one compound experiment. The worms of the same ordinal rank in the different divisions of the

experiment were as nearly comparable as they could be on the basis of preliminary examination. All solutions had an initial resistance of 2,700 ohms. All were similarly treated. The worms that

TABLE VI

A SUMMARY OF EXPERIMENT 99 SHOWING A COMPARISON OF DIFFERENT KINDS OF CONDITIONING

All solutions had an initial resistance of 2,700 ohms; 23° during day; refrigerator after midnight (see p. 3).

Worm No.	Survival in Hours				
	I Hypotonic Sea Water	II *Procerodes* Extract	III *Planaria maculata* Culture	IV M/33⅓ Cane Sugar	V Parotid Gland
1	4.17	70.78	10.75	5.25	7.92
2	12.45	63.75	9.25	23.25	8.58
3	1.92	52.37	52.70	9.25	8.58
4	12.50	16.25	10.50	7.67	6.50
5	5.00	78.75	33.25	7.67	6.50
6	5.00	101.57	9.75	5.00	8.33
7	5.00	2.67	9.75	2.67	8.33
8	6.17	28.00	31.17	10.17	6.17
9	4.67	63.17	4.67	12.17	7.17
10	3.33	86.88	12.17	2.33	4.67
11	27.25	52.37	7.92	21.25	2.92
12	1.92	111.25	7.92	9.25	1.92
13	5.25	.....	12.50	8.92	7.92
14	3.92	45.00	1.67	10.50	3.92
15	3.92	11.50	1.67	10.50	8.67
16	7.67	1.67	10.67	5.00	1.67
17	2.67	112.00	1.67	7.40	3.92
18	6.17	111.67	12.17	12.17	8.00
19	8.67	57.00	34.25	15.67	8.00
20	4.67	.....	30.67	7.33	7.33
Mean	6.61	59.25	15.20	9.67	6.35

STATISTICAL COMPARISONS

		Probability
II*— I=	52.45	0.0000
III— I=	8.59	.017
IV — I=	3.06	.0038
V — I=	— 0.26	.86
II*—III=	45.71	.0002
III—IV=	5.53	0.128

*Eighteen pairs only.

lived past midnight of the first day of the experiment (12.08–12.75 hours) were unduly favored, since they were left in a refrigerator until morning to check the processes leading to cytolysis.

Under the conditions of this experiment the *Procerodes* exposed to parotid-gland solution survived about the same time as did those in the hypotonic sea-water control. Those in M/$33\frac{1}{3}$ cane sugar lived some 3 hours longer (probability 0.0038). The worms isolated into *Planaria maculata* culture water survived 5.53 hours longer than those in cane sugar (probability 0.128); those in *Procerodes* water extract lived 45.71 hours longer still (probability 0.0002).

In this experiment the *Procerodes* clearly survived longer in the homotypic extract than in the heterotypic culture water. An absolutely opposed indication was reached in similar experiments in the preceding studies, the results of which are relisted along with others in the general comparative synopsis given in Table VII. Thus, in comparison No. 5 of that table, the earlier results on a unit of five experiments involving a hundred *Procerodes* show that in the heterotypic culture water the isolated marine flatworms survived 25.8 hours longer than did simultaneously tested worms in homotypic extract. The statistical probability of 0.028 indicates that the results are to be seriously considered, although they are not statistically so significant as are the results of Experiment 99 in the opposite direction. These latter are listed as comparison No. 8 of Table VII. Here we have the interesting condition of two separate series of paired experiments performed under generally similar conditions yielding opposing results that statistically are to be taken fairly seriously. When all the comparable tests are considered, the evidence to date indicates that neither is correct. The closely comparable data are summarized in comparison No. 4, which shows that an average of the thirteen tests made to date on this subject gives a longer survival time of 3.4 hours in the planarian culture water than in the homotypic *Procerodes* extract. The difference has a statistical probability of 0.696, which means that there are about seven chances in ten of obtaining as great a difference by random sampling. Hence, despite the positive indications shown on the one hand in comparison No. 5 and on the other in comparison No. 8, the data to date do not allow us to come to a positive conclusion regarding the relative merits of these two methods of conditioning hypotonic water.

Similarly, if we undertake to compare the relative effectiveness of these two kinds of conditioning by comparing the survival in each

with the survival time of animals tested simultaneously in paired experiments in plain hypotonic sea water, comparisons given in Table VII show that there is conflicting evidence, and we are again brought to the conclusion just reached from the consideration of the much more reliable direct comparisons.

TABLE VII

SOME OF THE AVAILABLE COMPARISONS IN SURVIVAL OF *Procerodes*, OTHER CONDITIONS BEING EQUAL, IN HOMOTYPICALLY AND HETEROTYPICALLY CONDITIONED MEDIUM

Survival time is given in hours. The worms survived longer in the medium given first in each comparison.

Comparison No.	Conditions Compared	Date of Experiments	No. of Experiments	Approx. No. of Worms	Difference in Survival (Hours)	Probability
1.....	*Procerodes* extract vs. Hypotonic sea water	1928 and 1931	18	810	15.81	0.0052
2.....	Planarian culture water vs. Hypotonic sea water	1928	19	386	22.86	.0002
3.....	Planarian culture water vs. Hypotonic sea water	1931	34	693	1.31	.00096
4.....	Planarian culture water vs. *Procerodes* extract	1928 and 1931	13	251	3.4	.696
5.....	Planarian culture water vs. *Procerodes* extract	1928	5	100	25.8	.028
6.....	*Procerodes* extract vs. Hypotonic sea water	Exp. 99 1931	1	18	52.45	.0000
7.....	Planarian culture water vs. Hypotonic sea water	1931	1	20	8.59	.017
8.....	*Procerodes* extract vs. Hypotonic sea water	1931	1	18	45.71	0.0002

Such considerations clearly demonstrate the necessity of obtaining a large number of mutually supporting results before one can arrive at definite conclusions in experimentation where so many variables are involved as in the present type of experimentation. One cannot doubt but that the results which show a high degree of statistical significance have real significance; the question is whether this is due to the factors supposedly being experimented upon or to other un-

recognized and therefore uncontrolled factors. Even so, the large amount of mutually supporting data that has been accumulated in the course of the experiments reported here and in previous papers on this subject leave small room for doubt as to the reality of the conditioning processes used being beneficial for *Procerodes* worms isolated into biologically conditioned hypotonic water. There is good evidence, also, that both homotypic and heterotypic conditioning are effective and that increasing the osmotic pressure, whether by the addition of sufficient sea water or of cane sugar, is also beneficial. The comparisons made in Table VII discourage attempts toward more definite statements about the mechanisms involved or the relative importance of different kinds of conditioning pending the development of more precise methods of experimentation and the more exact control of the physiological state of the animals being experimented upon.

GENERAL COMPARATIVE STUDIES

The general relation of these results to other similar phenomena have been discussed at length elsewhere (Allee, 1931). Since that manuscript was closed, Pantin and his associates (1931) have recorded a seres of studies upon the adaptation of *Gunda ulvae* to salinity. These have a bearing on the work just reported, both because *Gunda* is closely related taxonomically to *Procerodes* and because it is restricted to a sandy estuarine habitat which may range from full-strength sea water to almost completely fresh water. The reaction of *Gunda* to fresh water varies with the chemical composition of the latter. The worms swell less and recover more completely on their return to sea water if placed in fresh water rich in calcium rather than in calcium-deficient waters.

Conductivity measurements showed that *Gunda* transferred to fresh water began losing electrolytes immediately. Worms treated so take up water at once, as is shown by their swelling. The progressive series of changes upon transfer to fresh water appears to be similar to those given by *Procerodes* under similar conditions. As the worms swell, both lose the power to crawl; then, in most cases, they lose the power to retain their foothold and lie swollen greatly and ventrally concave. Later, activity ceases even when stimulated. In the most severe fresh-water tests (distilled water excepted) *Gunda* swelled to about 185 per cent of the normal volume, and the animals

died within 48 hours of continuous exposure. In distilled water, cytolysis began in about an hour.

These conductivity studies showed that *Gunda* is not a simple osmotic machine. For example, in a mixture of sea water plus Plymouth tap water, which caused 150 per cent swelling, the increase is of the same order as that which would be caused by osmotic inflow; in lower concentrations, the increase in size is far less than that which is expected on this account. Evidence that the tissues are working to keep down the swelling comes both from an increase in respiration found in *Gunda* and other marine animals when in fresh water and in the fact that reagents which depress the respiratory-rate break down resistance to fresh water. The experiments with ethyl alcohol and urethane with *Procerodes* do not show a hastening of cytolysis, though they do fail to give protection from fresh water. It is probably significant in this connection that the *Procerodes* long in the laboratory have distinctly less resistance than do those freshly collected, even when the laboratory stock is not greatly crowded and is kept in running sea water.

The processes involved in salt leakage in *Gunda* were found to be highly variable and may vary as much as 20 per cent in the same lot of worms at different times. Worms that have lived in sea water for a long time have a faster rate of salt loss than if transferred from sea water to fresh water and *vice versa* for a few times. This is in keeping with the large amount of variation found in different individuals of *Procerodes*.

Of the factors affecting salt loss in *Gunda*, Pantin found, as have I with survival of *Procerodes* in fresh water, that pH is not effective at the ranges tested. Glycerol of the same osmotic pressure as stream water is no more effective with *Gunda* than is distilled water. Dilute NaCl (0.004 M) is less efficient in maintaining the inner salt content than is $CaCO_3$ and $CaCl_2$; hence, direct osmotic pressure is not the only factor involved. The analyses Pantin and his associates have made indicate that *Gunda* lives in osmotic equilibrium with sea water and comes to be in a steady physiological state with certain fresh waters, but that the steady state in the latter is not the result of simple osmotic equilibrium; furthermore, it cannot be continued indefinitely. This steady state is maintained in part as a result of work done which finds an expression in increased rate of respiration.

The effect of the presence of biologically conditioned water upon such work is not known, We do have good evidence (Allee, 1929) that *Procerodes* loses electrolytes less rapidly into water of similar resistance if it has been biologically conditioned. This means that the observed protection is not the result of a more rapid leaching of electrolytes into the protective media with the closer approach to osmotic equilibrium that would result. Apparently, both leakage of electrolytes and intake of water is lessened by the presence of the biologically conditioned medium. The latter is shown by the frequently longer retention of normal body form and powers of locomotion, without, of necessity, considering the greater delay of cytolysis in these conditioned media. There is also the possibility that the presence of the materials responsible for biological conditioning defers the onset of cytolysis for some reason not directly associated with water or salt exchange for the worm as a whole.

These problems centering about the causes of protection for *Procerodes* in these conditioned solutions include, then, the effect of the biological conditioning upon the physiological state of the limiting membranes involved in water intake and salt outgo. They are also concerned with the relative effect of exposure to conditioned and unconditioned media upon physiological processes which probably find some expression in rate of respiration, as well as the more obvious and direct effects upon processes of osmosis. The analysis of these general physiological aspects of the problem has scarcely begun.

SUMMARY

1. Previous experiments (Allee, 1928, 1929) concerning the action of hypotonic water extracts of other *Procerodes* and *Planaria maculata* culture water on the marine *Procerodes wheatlandi* isolated therein, are confirmed.

2. Adsorption on charcoal or on egg albumen does not significantly affect the protection furnished by planarian culture medium.

3. No protection is furnished by egg albumen, gum arabic, or mucus made by dissolving dried parotid gland in water. Previous work had shown the same situation with gelatine.

4. Ethyl urethane, a depressant, does not give protection; it acts in this respect as had ethyl alcohol in previous experiments.

5. Water solutions or suspensions made from ether-soluble and ether-insoluble fractions of *Procerodes* extracts exhibit less protection than does the whole water extract.

6. Cane-sugar solutions definitely confer protection in all concentrations tried from M/1 to M/33⅓. Solutions of M/40 and M/50 gave the same survival as did the non-sugar controls. The protection conferred in these experiments by M/25 and M/33⅓ solutions was approximately that given in accompanying isolations into planarian culture water.

7. The *Planaria maculata* culture water did not possess osmotic pressure beyond that furnished by electrolytes, and these were balanced with those in the controls in all this series of experiments in so far as that can be done by testing their conductivity.

8. These experiments reinforce and extend the essential conclusions of the earlier work but do not throw light on the nature of the protective mechanisms involved.

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EFFECTS OF POPULATION DENSITY ON THE RATE OF REPRODUCTION IN OXYTRICHA[1]

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INTRODUCTION

THE effects of varying the number of Protozoa in a given volume of culture medium and of varying the volume of culture medium for a given number of Protozoa have been investigated by a number of workers in recent years. It is generally recognized that Woodruff (1911*a*) was the first to carefully study this problem. He found a decrease in the rate of reproduction with an increase in the density of the population: "The rate of reproduction of *Paramecium aurelia* and *Paramecium caudatum* is influenced by the volume of the culture medium within the limits tested and the greater the volume the more rapid is the rate of division" (1911*a*, p. 100). He concluded that these results were caused by toxic excretory substances, produced by the organisms themselves, which are more effective in small volumes of culture medium.

This problem remained as Woodruff left it until 1921*a* and 1921*b*, when T. B. Robertson described opposite results with two infusorians, *Enchelys farcimen* and *Colpidium colpoda* (later identified as *Colpoda cucullus*). He found that when two Infusoria (*Enchelys* or *Colpoda*) are introduced into a small amount of fresh culture medium, the early rate of reproduction is more than double that of a single individual of the same species in the same volume of culture medium. This increased rate of reproduction has been designated the "allelocatalytic effect" by Robertson. Allee (1931, p. 166) gives the following statement of Robertson's hypothesis:

> During nuclear division each nucleus retains the charge of autocatalyst with which it was provided, and adds to it during the course of nuclear synthesis. At each division the autocatalyst is shared between the nuclear substance and the surrounding medium in a proportion determined by its relative solubility and by its affinity for chemical substances within the nucleus. The mutually ac-

[1] The writer is greatly indebted to Dr. W. C. Allee for suggesting this investigation and for facilities and advice given in connection with it.

celerative or allelocatalytic effect of contiguous cells is due to each cell's losing less of the autocatalyst to the medium because of the presence of the other. According to this hypothesis, the autocatalytic effect of growing colonies, whether attached or detached, is due to the same cause.

Robertson, in his later papers, stated that this effect is more marked when the organisms have been freed from contamination with the parent culture medium by repeated washings. In his last paper he stated that all of his data and conclusions concerning allelocatalysis in Infusoria were valid except that they might apply to the food organisms. "Whether the allelocatalytic effect originates with the ciliates, or with associated food organisms, however, it remains a mutually accelerative effect of contiguous organisms upon their reproductive rates" (1927, p. 5).

Robertson's results aroused considerable interest; and because of this, several investigators have made similar studies, using different organisms. Cutler and Crump (1923*a* and 1923*b*), using *Colpidium colpoda*, report that they could not obtain the effect described by Robertson. In a like manner Greenleaf (1924, 1926), using *Paramecium aurelia*, *P. caudatum*, *Pleurotricha lanceolata*, and *Stylonychia pustulata;* Calkins (1926), using *Uroleptus mobilis;* Myers (1927), using *Paramecium caudatum*, and Jahn (1929), using *Euglena*, report that they were unable to duplicate the results described by Robertson. On the other hand, Yocum (1927, 1928) reported results similar to those of Robertson, from his work with *Oxytricha*. Peterson (1929) found that she could obtain a decrease or an increase in the relative rate of reproduction of associated and isolated *Paramecium caudatum* by varying the volume of culture medium. When she used $\frac{5}{2^4}$ cc. of medium or less, the two-animal cultures reproduced no faster than a single one in 24 hours. When she used $\frac{20}{2^4}$ cc. of medium, the two-animal cultures reproduced faster than the one-animal cultures. All of the work mentioned in this paragraph has been carefully reviewed by Allee (1931).

Grimwald (1928) found that there was no difference in the division-rate of *Colpidium colpoda*, using one, two, and three organisms in volumes of hay infusion varying from 12.5 to 90 cubic millimeters. In smaller volumes (0.08–12.5) the division-rate was slower than in the larger volumes.

Chejfec (1929) studied the bacterial requirements of *Paramecium caudatum* for the maintenance of life. He states that in a concentration of 24,000 *Bacillus coli* per cubic millimeter a single animal has a normal division-rate. Using concentrations of bacteria much less than the amount needed to give a normal division-rate in *Paramecium*, he reports no evidence of Robertson's effect. He did not investigate the effect of greater concentrations of bacteria on the rate of reproduction of *Paramecium*, using one and two organisms in the same volume of medium.

Darby (1929, 1930) found that *Paramecium caudatum*, *P. aurelia*, and *Stylonychia pustulata* each have an optimum pH for growth and that each organism tends to buffer or bring its medium to its optimum pH. He suggests (1930, p. 312) that

> a possible explanation of Robertson's results appears to be that his medium was away from the optimum pH, and very weakly buffered. The organisms tended to change it, and the more he put in at a time, the quicker the improvement. In the smaller volumes it was easier to bring this about than in the larger ones. In too small a volume, however, the accumulation of ammonia would soon retard the growth; while in the very large volumes the buffering power of the organisms would be ineffective.

In all of the investigations referred to above, only those of Cutler and Crump and Chejfec describe attempts to control the kinds and numbers of bacteria in the culture medium used. The importance of controlling the food supply in such investigations has long been recognized. Jennings (1908) insisted that the bacteria in the culture medium must be the same in any series of experiments. He made no attempt to use pure cultures of bacteria. He took *Paramecia* from vigorous stock cultures, washed them in large amounts of culture medium, and used this washing fluid to seed the medium used for the growth of pure lines of *Paramecium*. He states that, by doing this every few days, he was able to maintain the bacterial content of his cultures at an optimal efficiency as to food.

Hargitt and Fray (1917) plated out bacteria from their protozoan cultures and used pure lines of bacteria in their experimental cultures. Eight of these used singly as food for *Paramecium* were unsatisfactory. *Bacillus subtilis* used alone as food for *Paramecium* proved to be quite satisfactory. Yet they concluded that cultures

of mixed bacteria are, as a rule, better for the growth of *Paramecium* than any single kind of bacteria. They suggest that by using mixtures of known bacteria in sterile infusions better results can be obtained than by using the ordinary mixed cultures of unknown bacteria.

Phillips (1922) found that *Paramecium aurelia* grew better on her A′ and C′ strains of bacteria than on C′ alone. They would not grow on A′ alone or on nine other kinds of bacteria or any combinations of them.

Philpott (1928) reports that *Paramecium* will grow on hay infusions containing only a single kind of pathogenic bacteria, *Bacillus pyocyaneus*. He was not able to obtain growth of *Paramecium* using *Bacillus enteritidis*.

Luck, Sheets, and Thomas (1931) studied the rôle of bacteria in the nutrition of *Euplotes taylori*. Using *Bacillus coli* and five other strains of bacteria alone, they found no favorable growth. When they used two strains of bacteria in the culture, good growth occurred. They later reported that good growth was obtained by using a pure culture of *B. fluorescens pseudomonas*. Barker and Taylor (1931) found that suspensions of a pure culture of the bacterium *Pseudomonas fluorescens* in a balanced physiological medium served as a very suitable food for the ciliate, *Colpoda cucullus*.

Losina-Losinsky (1931) studied the feeding reactions of *Paramecium caudatum*. The results show that bacteria form the chief food substance of *Paramecium*, and that some bacteria are taken in in greater numbers than others. *Paramecium* was found to react quite positively to suspensions of *Bacillus subtilis*, less positively to suspensions of *B. fluorescens liquefaciens*, and very slightly to suspensions of *B. coli communae*.

Stuart and Banta (1931) report that they have been able to grow the cladoceran, *Moina macrocopa*, successfully on suspensions of *Aërobacter aërogenes* introduced into their normal dilute manure infusions. In their work they found that the sex of the young produced by experimentally crowded *Moina macrocopa* mothers can be controlled by the amount of available bacteria present in the medium. In cultures with large numbers of bacteria, the mothers produced only female young. As the number of bacteria was decreased, males

were produced in direct proportion to the decrease in the bacterial count of the medium. From the standpoint of this investigation their work is especially interesting because they found an optimal density of bacteria for maximal reproduction. The greatest number of young was produced, not in the bottles containing the greatest number of bacteria, but in the bottles containing fewer bacteria wherein the sex ratio was approximately three females to one male. At the same time Stuart, McPherson, and Cooper (1931) developed a technique for sterilizing *Moina*. In this part of the work they found that *Moina* requires living bacteria as food, and that all species of bacteria do not serve equally well as food for Cladocera. Some of the bacteria used were actually toxic to *Moina*.

The purpose of the present investigation has been to study the effects of population density on the rate of reproduction in *Oxytricha*, using (1) the common hay infusion with unknown mixed bacteria and (2) a non-nutritive balanced physiological medium in which the kinds and number of bacteria can be controlled.

MATERIALS AND METHODS

Oxytricha fallax was used in all of the experiments. Stock cultures were kept in 200 cc. bottles. Hay infusions were used for the stock cultures. These were prepared, in the initial experiments, by boiling 5 grams of timothy hay in a liter of tap water for 10 minutes. An infusion made with 2 grams of hay proved to be quite suitable and was used for stock cultures in the second phase of the work. The infusion was filtered, when cool, and inoculated with 1 cc. of the preceding culture. Several new stock cultures were started each week, so that a number of flourishing cultures were always on hand. Infusions for the isolation cultures were prepared in the same way, and 200 cc. of the fresh infusion were inoculated with bacteria from a flourishing stock culture of *Oxytricha* 6 hours prior to use. For this purpose 1 cc. of the stock culture fluid, free from *Oxytricha*, was added to the 200 cc. of fresh infusion.

In all of the experiments ground-glass protozoan isolation dishes were used as containers. Evaporation in these small cultures was maintained at a minimum by keeping the dishes in moist chambers. The experiments reported in Tables II–VII were run at room tem-

peratures. All of the other experiments were carried out in a constant temperature room at 27° C., except as noted. In this room the humidity was also high, as a further prevention of evaporation.

Experimental animals were obtained from pure-line isolation cultures. New pure-line cultures were started from stock cultures every 2 weeks. Pure-line cultures containing 32 sister-organisms were used in all experiments. Thus, the organisms used in all paired experiments came from the same isolation culture which started as a single individual. Care was taken not to use either the very small animals or the very large ones ready for division.

The organisms were isolated by means of drawn-out pipettes and were washed twice in 5 drops of fresh medium before they were transferred to the experimental dish. In all of the isolation experiments where hay infusion was used, 24 drops were equal to 1 cc. In the later experiments 18 drops (as reported in Tables X, XII, XIII, XIV, and XV) and 16 drops (as reported in Table XI) were equal to 1 cc.

The hydrogen-ion concentration of the fresh infusions varied from pH 6.6 to pH 7.2, with the greater number of the infusions having an initial pH of 6.8. The hydrogen-ion concentration was determined by the colorimetric method. In those cases where the hydrogen-ion concentration of small cultures was determined at the end of the experiment, the determination was made by a comparison of the unknown with commercial standards in vials of about $\frac{1}{2}$ cc. capacity.

In the second phase of the work, suspensions of bacteria were used in non-nutritive balanced physiological medium. These suspensions were made by removing the bacteria from peptone-glucose-agar plates with a sterile platinum loop and introducing the bacteria into the sterile physiological medium. This medium, originally used by Osterhout (1906) and recently used by Barker and Taylor[2] (1931) in their study of *Colpoda*, is given in Table I.

This solution was diluted two hundred twenty-five times to the approximate concentration of pond water, or 0.012 per cent total salt. One cc. of M/20 NaH_2PO_4 was added to each 30 cc. of this

[2] The author wishes to express his appreciation of the help and advice given by Dr. C. V. Taylor and Mr. H. A. Barker in the development of this technique and in the use of it.

diluted solution for buffering, and the hydrogen-ion concentration was adjusted with small amounts of M/20 NaOH.

In the cases where suspensions of bacteria were used in sterile media, the *Oxytricha* were sterilized by numerous washings in sterile medium, as described by Hargitt and Fray (1917) and Parpart (1928), before they were introduced into the experimental dishes. All dishes and pipettes used in these experiments were sterilized each time before used.

TABLE I

BALANCED PHYSIOLOGICAL MEDIUM

(2.7 per cent total salt [0.375M])

0.375M	Parts
$NaCl$	1.000
$MgCl_2 \cdot 6\ H_2O$	78
$MgSO_4 \cdot 7\ H_2O$	38
KCl	22
$CaCl_2$	10

Robertson (1924*c*) emphasizes that in experimental work of this kind care must be taken to estimate the population sometime before it has reached the maximal density. In the preliminary experiments in this work, counts were made every 6 hours. On the basis of these results, it seemed desirable to take the counts at the end of 24-hour periods for comparison.

EXPERIMENTS USING HAY MEDIUM

The first set of experiments was run using *Oxytricha* isolated singly and in two's into 1 drop of bacterized hay infusion. Table II shows typical results. At the end of 24 hours there was no difference in the rate of reproduction. However, at the end of 42 hours the fission-rate had slowed down in the two-animal cultures. In no case, in the two-animal cultures, was the total number of animals double that of the one-animal cultures at the end of 42 hours. This seems to indicate that the maximal density had been reached in these cultures sometime between 24 and 42 hours.

A similar set of experiments, using 2 drops of bacterized hay infusion, was carried out. Table III gives the results of a typical series. In this set, likewise, there was no difference at the end of 24

hours between the cultures started with one and with two animals. In half of the cases there were a few more organisms in the original one-animal cultures, and the opposite of this in the other half.

Petersen (1929) reported the same kind of results for her experiments with *Paramecium*, using small volumes of medium. As she

TABLE II

SHOWING THE RESULTS OF 1-DROP EXPERIMENTS

	24-HOUR COUNT		42-HOUR COUNT	
	1-Animal Seeding	2-Animal Seeding	1-Animal Seeding	2-Animal Seeding
Exp. A	4	8	16	25
Exp. B	7	13	31	34
Exp. C	8	16	26	33
Exp. D	7	16	31	41
Exp. E	8	16	18	35

TABLE III

SHOWING THE RESULTS OF 2-DROP EXPERIMENTS

	24-HOUR-COUNT	
	1-Animal Seeding	2-Animal Seeding
Exp. A	8	18
Exp. B	4	9
Exp. C	8	15
Exp. D	7	13
Exp. E	11	24
Exp. F	7	12

had been able to obtain different results using larger volumes, it was deemed necessary to carry out several sets of experiments using larger volumes. *Oxytricha*, isolated singly and in two's into 5 drops of bacterized hay infusion, were used in the experiments reported in Table IV. There were fourteen series in this set of experiments. The number given after each series in this and later tables is the average number of animals produced from a single organism in 24 hours. In each series four dishes with two animals in each and eight dishes with one animal in each were used. Thus, the same number of animals was used in each condition; half of them were isolated

singly, and half in two's. This arrangement was not used in the experiments reported in Tables II and III, but was used in all of the other experiments, as it offered a better basis for comparison. Each series was carried out on a different day. The conditions of the experiments were kept the same except as to temperature. The whole set of experiments was run during the very hot month of August, 1930. The temperature in the laboratory (Whitman Laboratory) reached 34°–35° C. almost every day. The very high rate of reproduction reported in these experiments must be associated with the very high temperatures. The experiments in Series 14, carried out in much lower temperatures, show a marked decrease in the rate of reproduction. Other experiments, to be referred to later, show the effects of temperature on the rate of reproduction.

TABLE IV

RESULTS OF 5-DROP EXPERIMENTS

	1-Animal Seeding	2-Animal Seeding
Series 1	17.6	22.5
Series 2	19.1	21.5
Series 3	14.1	16.9
Series 4	30.7	36.4
Series 5	43.0	48.7
Series 6	40.1	37.2
Series 7	23.6	25.1
Series 8	33.9	45.6
Series 9	41.8	44.8
Series 10	18.1	17.7
Series 11	18.6	24.8
Series 12	30.4	39.4
Series 13	18.9	20.1
Series 14	6.9	7.8

An examination of the data in Table IV shows that the average rate of reproduction of the doubles was greater than that of the singles in twelve of the fourteen series. "Student's" method (Student, 1925; Fisher, 1925) of statistical analysis may be applied to such paired experiments to determine their possible statistical significance. Such analysis shows that with the results given in Table IV there are 32 chances in 10,000 of random sampling giving such a wide deviation from the mean. This is well within the range of statistical significance.

Ten drops of bacterized hay medium were used as cultures for a similar set of experiments. The results are given in Table V. The temperature range given is that recorded from a thermometer in the laboratory. These readings show only the temperature range during the day. No attempt was made to obtain the temperatures at night. The effect of temperature on the reproduction-rate is again clearly shown. The data in Table V show that in nine of the fourteen series there was a higher average rate of reproduction with an initial seeding of two as compared with cultures started by a single individual.

TABLE V

RESULTS OF 10-DROP EXPERIMENTS

	1-Animal Seeding	2-Animal Seeding	Temperature Range in Degrees Centigrade
Series 1	21.2	23.5	27–35
Series 2	14.9	16.2	28–33
Series 3	11.6	11.4	26–33
Series 4	9.4	12.9	28–31
Series 5	10.6	12.4	25–30
Series 6	13.2	13.2	27–30
Series 7	19.2	22.4	30–35
Series 8	7.7	7.5	25–30
Series 9	6.9	9.5	25–29
Series 10	7.0	6.6	23–29
Series 11	6.6	6.5	23–28
Series 12	6.6	6.8	23–27
Series 13	7.2	7.4	24–28
Series 14	6.2	6.5	23–26

In one series the average rate was the same, and in the other four the one-animal experiments had a slightly higher average rate. A statistical analysis of these data shows that there are 15 chances in 1,000 of random sampling giving such a wide deviation from the mean. This would generally be taken as significant.

A set of experiments using single animals in 1, 2, 5, 10, and 20 drops of hay medium was conducted. Seven series of these experiments were carried out, using four cultures for each volume mentioned. The averages for all of these gave 33, 32, 28, 23, and 22 as the average number of animals respectively in the 1-, 2-, 5-, 10-, and 20-drop cultures at the end of 24 hours. There was not a significant difference in the 1- and 2-drop cultures. The highest rate of repro-

duction was found in the 1- and 2-drop cultures, with a progressively decreasing rate in the other volumes.

This set-up was repeated using 2-, 5-, 10-, 20-, and 40-drop cultures. Fourteen series were run, using four cultures of each volume in each series. Table VI gives the average results from these experiments. The results of this set of experiments were, in general, the same as those of the preceding set. The highest rate of reproduction

TABLE VI

RESULTS AFTER 24 HOURS OF ISOLATING SINGLE ANIMALS INTO 2-, 5-, 10-, 20-, AND 40-DROP CULTURES

	No. of Drops				
	2	5	10	20	40
Series 1	10.5	9.5	6.0	6.0	5.0
Series 2	7.8	6.2	5.5	4.8	4.5
Series 3	4.8	4.8	4.2	4.0	3.0
Series 4	8.0	7.5	6.5	4.2	3.5
Series 5	6.2	6.7	5.7	4.0	3.5
Series 6	5.7	4.5	4.0	3.2	2.8
Series 7	8.0	7.0	7.0	6.5	6.0
Series 8	6.5	5.2	5.2	5.0	3.5
Series 9	5.0	5.0	4.0	3.5	2.8
Series 10	6.5	5.2	5.2	4.0	3.5
Series 11	5.8	4.5	4.0	4.0	3.5
Series 12	3.5	3.5	3.0	3.0	2.5
Series 13	3.0	2.5	2.0	2.0	1.8
Series 14	8.0	6.5	5.0	3.2	3.0
Mean	6.3	5.6	4.8	4.1	3.5

is found in the 2-drop cultures, with a general decrease with each increase in the volume of the medium. When "Student's" method is applied to the data in Table VI, it is found that there is a significant difference in the average reproduction-rate with each increase in the volume of the medium. That is, the average rate of the 2-drop cultures is higher, and significantly higher, than that of the 5-drop cultures; the average rate of the 5-drop cultures is significantly higher than that of the 10-drop cultures; and so on.

All of the experiments described above were carried out at room temperatures. At this point in the experimentation a constant temperature room with controlled high humidity was made available, and all of the remaining experiments were made with the tempera-

ture controlled. The temperature was kept at 27° C. (unless noted otherwise), and at no time was the variation from this more than 1°. As a check on the previous experiments, a set was carried out, using single animals in volumes of 2 and 20 drops, with the temperature constant. In this set ten cultures for each volume were used in each series. Table VII shows the average results. The maintenance of a constant temperature did not change the type of results obtained. In this set of experiments it is obvious that rate of reproduction in

TABLE VII

RESULTS AFTER 24 HOURS OF SEEDING SINGLE ANIMALS INTO 2- AND 20-DROP CULTURES UNDER CONSTANT TEMPERATURE AT 27° C.

	No. of Drops	
	2	20
Series 1	7.0	5.1
Series 2	3.6	2.9
Series 3	4.2	3.1
Series 4	5.7	3.7
Series 5	3.1	1.8
Series 6	3.1	1.8
Series 7	6.0	3.3
Mean	4.7	3.1

the 2-drop cultures was, in each series, higher than that in the 20-drop cultures.

Maupas (1888), Middleton (1918), Jollos (1921), Dawson (1928), and others have studied rather thoroughly the effects of temperature on the fission rate of various ciliates. They have found that raising the temperature generally causes an increase in the reproduction-rate. This effect, while aside from the main problem, has been noticed in these experiments. This suggested that it would be interesting to compare the results of two sets of experiments like the one given in Table VII, running one set at 70° F. and the other at 80° F., with the temperature constant. Accordingly, such an experiment was made. A comparison showed the following: (1) that the average reproduction-rate of the 2-drop cultures was highest in both sets, (2) that the average reproduction-rate of the 2-drop cultures at 80° F.

was higher than that of the 2-drop cultures at 70° F., and (3) that the average rate of reproduction in the 20-drop cultures at 80° F. was, in most cases, higher than that of the 20-drop cultures at 70° F. This completes the demonstration that the difference obtained in the reproduction-rate in previous experiments is independent of temperature.

Darby (1930) states that he obtained the best results in his work when he used culture dishes coated with paraffin. He suggests that small amounts of toxic materials may leach out of the glass and affect the organisms. It seemed desirable to see if the failure to use paraffin-lined dishes was the cause of the differences in reproduction-rate

TABLE VIII

SHOWING THE EFFECTS UPON THE RATE OF REPRODUCTION OF ANIMALS ISOLATED INTO PARAFFIN-LINED DISHES

	PLAIN		PARAFFIN-LINED	
	2 Drops	20 Drops	2 Drops	20 Drops
Average.....	7.1	5.4	8.4	6.4

already found and described above, as no paraffin-lined dishes had been used in any of the experiments up to this point. Two parallel sets of tests were run—one in plain dishes and the other in paraffin-lined dishes—each seeded with single animals placed in 2- and 20-drop cultures. There were nine parallel series in each set with four cultures for each volume in each series. The average numbers of animals at the end of 24 hours were averaged to give the figures reported in Table VIII.

These results show a higher average rate of reproduction in the paraffin-lined dishes. However, the difference is of doubtful significance. When the 2-drop cultures of the plain and paraffin-lined dishes are compared, there are 17 chances in 100 of random sampling giving such a wide deviation from the mean. When the 20-drop cultures of the plain and paraffin-lined dishes are compared, there are 29 chances in 100 of random sampling giving such a wide deviation from the mean. But the important thing, from the standpoint of this investigation, is that the 2-drop cultures in both the plain and paraf-

fin-lined dishes showed a significantly higher rate of reproduction. As it is difficult to keep the paraffin-lined dishes clean and in good condition, and as their use does not seem to affect the type of results already obtained, it was not considered necessary to use them in all of the future experiments.

Darby (1930), as was mentioned earlier in this paper, found that each of the three ciliates which he studied had an optimum pH for reproduction. Also, he suggests that the type of results found by Robertson might be due to the use of a medium with a hydrogen-ion concentration quite removed from that which is the optimum for reproduction in the infusorian used; under these conditions an infusorian, which he says tends to bring its medium to its optimum pH, placed in the smaller of two volumes, would change the hydrogen-ion concentration faster than would one placed in a larger volume, and thus the difference in reproduction-rate might be accounted for. In view of Darby's suggestion it seemed desirable to study the reproduction-rate of *Oxytricha* in cultures with different hydrogen-ion concentrations. In this work, Darby's method of adjusting the pH of the hay medium was used. This was accomplished by adding 1 cc. of M/20 NaH_2PO_4 to 30 cc. of the hay medium for buffering, and adjusting the pH with suitable quantities of M/20 NaOH. A set of experiments was run, using solutions with initial pH's of 6.0, 6.4, 6.8, 7.2, 7.6, and 8.0. As a control unbuffered hay medium, with a pH of 6.8, was used. In these experiments single animals were isolated into 5 drops of the medium. Eight series were run with four cultures for each pH range in each series. The pH of each culture was measured at the end of 24 hours, when the count was made. Thus, Table IX gives the pH range for the different cultures. Paraffin-lined dishes were used in these experiments.

Each number in Table IX represents the total number of organisms produced from four animals in 24 hours. These data show that the highest rate of reproduction occurred in the control cultures and in the cultures with an initial pH of 6.4. However, the differences are not consistent, and it cannot be stated that there is a very definite optimum indicated in the range used. There is much evidence to show that, while some protozoans have a very narrow pH tolerance, others have a very wide pH tolerance (Wang, 1928). It is indicated

in these experiments that *Oxytricha* belongs to the latter group. Darby (1930) found that, while there was a very marked optimum pH for *Paramecium aurelia* and *P. caudatum*, this was not true for *Stylonychia pustulata*. He suggests that the hypotrichs have a wide pH tolerance.

A sufficient number of experiments were carried out, using single animals in 2- and 20-drop cultures with the hydrogen-ion concentration varied from pH 6.0 to 8.0, to indicate that a difference in the pH of cultures, within these limits, would not change the type of results already obtained. Within this range, the rate of reproduction in the 2-drop cultures was always higher than that in the 20-drop cultures.

TABLE IX

RESULTS OF VARYING THE HYDROGEN-ION CONCENTRATION WITH SINGLE ANIMALS ISOLATED INTO 5 DROPS OF MEDIUM

	pH RANGE						
	6.0–7.2	6.4–7.4	6.8–7.6	7.2–7.8	7.6–8.0	8.0–8.4	Control 6.8–7.8
Series 1	24	27	17	20	12	14	20
Series 2	32	31	30	32	26	38	28
Series 3	22	26	20	14	14	16	18
Series 4	20	28	20	20	14	21	25
Series 5	22	21	18	16	11	16	20
Series 6	29	29	32	32	25	27	30
Series 7	29	32	30	32	29	20	47
Series 8	14	24	24	20	18	14	30
Av.	24	27	24	23	19	21	27

With a difference in division rate in different population densities clearly indicated by the experiments already reported, and with no obvious cause for the difference, except the volume relations of the animals and culture medium, it seemed desirable to study the food of *Oxytricha* and to find out if the food organisms have any part in causing the difference in reproduction-rate in different population densities.

As the first step in this study, several agar plates were inoculated with some of the liquid from a thriving culture of *Oxytricha*. At the

end of 48 hours each of these plates contained numerous colonies of different kinds of bacteria. Pure cultures of the bacteria from seven of the predominating colonies were grown on agar plates. The ability of *Oxytricha* to grow and reproduce in cultures with a single kind of bacteria as the source of food was tested. Only the predominating kinds of bacteria were used in these tests, because it was considered that the bacteria serving as food for Infusoria in a thriving culture would be fairly abundant in the culture medium. The seven kinds of bacteria isolated in pure cultures were designated as "G," "R," "T," "W," "Y," "S," and "B." "B" was identified as *Bacillus subtilis*. Mr. Barker, who was working in the laboratory at the time, suggested the use of *Pseudomonas fluorescens* which he had in pure cultures. This was done, making eight kinds of bacteria available for use in the food tests.

To determine the ability of *Oxytricha* to grow and multiply, with a single kind of bacteria as the food, necessitated the sterilization of the *Oxytricha* used. This was accomplished by the method of washing suggested first by Hargitt and Fray (1917), with the improvement suggested by Parpart (1928). Medium sterilized in an autoclave was used for the washing fluid. Transfers were made in individual sterilized pipettes. At the end of the twelfth washing, the animals were ready for the test. Five cc. of sterile hay medium in a small sterilized test tube were inoculated with a small amount of a pure culture of bacteria; and into this, five *Oxytricha*, sterilized by twelve washings, were introduced. Three such tests were made for each of the eight kinds of bacteria. The results were the same each time the tests were made. Each time at the end of a week there was a good growth in the cultures inoculated with *Bacillus subtilis* and with *Pseudomonas fluorescens*. In the cultures inoculated with the other types of bacteria the *Oxytricha* were dead at the end of 7 days, with the exception of those cultures inoculated with the bacteria designated as "R." The organisms in these cultures had divided a few times by the end of 14 days, but it was obvious that they were not in a favorable environment. These tests show two things: that at least two kinds of bacteria used alone serve as suitable food for *Oxytricha*, and that at least six other kinds used alone do not.

EXPERIMENTS WITH A NON-NUTRITIVE MEDIUM

In an analysis of the effect of bacteria on the rate of reproduction in *Oxytricha*, it seemed necessary to control the numbers of bacteria. This is not possible in the use of a nutritive hay medium. At the suggestion of Dr. C. V. Taylor, the use of a balanced physiological medium (given in Table I) diluted to the approximate concentration of pond water was used. In the beginning it was necessary to find out what concentration of bacteria proved most suitable for reproduction in *Oxytricha*. A number of different-sized platinum loops were made and a loopfull of bacteria was introduced into 10 cc. of the balanced medium. Suspensions of bacteria so made were used for culturing the *Oxytricha*, and the concentration giving the most satisfactory results was used for further experimentation. Such a test was made for both *Bacillus subtilis* and *Pseudomonas fluorescens*.

With the optimal density of bacteria (for the two kinds just mentioned) for favorable reproduction determined, a set of parallel experiments was run, using single *Oxytricha* introduced into a suspension of *Bacillus subtilis*, a suspension of *Pseudomonas fluorescens*, an equal mixture of the two bacterial suspensions, and into the ordinary hay infusion. Two drops were used in each case. The pH of the balanced medium was 6.8 at the beginning of the experiments. The pH was adjusted as outlined in the section on methods. This pH was used because it was the same as that of most of the hay infusions in the previous experiments and because the studies reported in Table IX showed this pH to be well within the range for good reproduction. In this, and in all other sets of experiments to be reported, the temperature was held at 27° C. Table X shows the results of these experiments.

Each number in Table X is the average number of animals produced in four dishes at the end of 24 hours, when each dish started with a single animal. These experiments show that there was a distinctly higher rate of reproduction in the suspensions of *Pseudomonas fluorescens* than in any other type of culture. Suspensions of *Bacillus subtilis* proved to be the least suitable. As might be expected, a mixture of the two was more suitable than a suspension of *B. subtilis* alone. In no case was the rate of reproduction in the hay infusion cultures nearly as high as that in the suspensions of *Pseudomonas*

fluorescens. It averaged about the same as the average rate in the mixed suspensions. This work clearly shows that suspensions of *Pseudomonas* are more suitable than ordinary hay infusions for this type of experimentation with *Oxytricha;* accordingly, suspensions of this kind of bacteria were used in the further analysis of the problem.

It seemed logical to suppose that bacteria introduced into the non-nutritive physiological medium would not increase in numbers. However, a check on this supposition was made. A standard suspension

TABLE X

SHOWING THE REPRODUCTION-RATE FOR 24 HOURS OF *Oxytricha* INTRODUCED SINGLY INTO THE BALANCED PHYSIOLOGICAL MEDIUM AND INTO THE HAY MEDIUM

	KIND OF MEDIUM			
	Suspension of *Pseudomonas fluorescens*	Suspension of *Bacillus subtilis*	Mixed Suspension	Hay Infusion
Series 1	8	2.2	3	5
Series 2	18.7	9.2	9	12
Series 3	16	5.2	11.5	9.2
Series 4	27.7	10.2	16.5	15.5
Series 5	21	11.2	13.7	10.7
Series 6	18.5	7.5	11	15
Series 7	11.5	4.5	6	8
Series 8	14	12	10	11
Series 9	28.7	13	18.8	18.5
Av	18.2	8.3	11.1	11.6

was made by introducing a loopfull of bacteria into 10 cc. of sterile balanced medium in a test tube. This standard suspension was the concentration which had been found to be most suitable for reproduction in *Oxytricha*. One cc. of this standard suspension was diluted 200,000 times with sterile balanced medium. One drop of this suspension (16 drops equal 1 cc.) was spread on an agar plate, and the colonies of bacteria formed after 48 hours were counted. The standard suspension was kept for 24 hours, and after the suspension was mixed thoroughly this process was repeated. By comparing the number of colonies formed at the beginning and at the end of 24 hours, one could determine whether the bacteria had increased in

numbers. Table XI gives the results of a number of these comparisons.

In this work the bacteria cultures were from 5 to 12 days old. In the first six series in Table XI each number is the average number of colonies found on four plates. In Series 7 each number is the average number of cultures found on twelve plates. All of the plates in each series were made from the same standard suspension. The data in Table XI show that there is some decrease in the number of bacteria in the balanced medium during 24-hour periods. On such evidence it was assumed that there would be little, if any, reproduction of the bacteria in similar suspensions containing the *Oxytricha*.

TABLE XI

SHOWING THAT THE BACTERIA SUSPENDED IN THE BALANCED MEDIUM DO NOT INCREASE IN 24 HOURS

	No. at the Start	No. at the End of 24 Hours
Series 1	37	30
Series 2	25	21
Series 3	34	28
Series 4	37	35
Series 5	40	32
Series 6	35	28
Series 7	31	27

If the numbers in Table XI, which represent average numbers of colonies formed from individual bacteria, are multiplied by 200,000 $\times$16, or 3,200,000, the average number of bacteria in 1 cc. of the standard suspension will be obtained. This number ranges from 80 to 128 million per cubic centimeter for the experiments reported in Table XI. During the course of the remaining experiments, nine series of bacterial counts similar to those just mentioned were made at different times. The average number of bacteria in millions per cubic centimeter of standard suspension were: 135, 100, 98, 118, 128, 133, 129, 113, 90, and 111. This range is practically the same as that found in Table XI. The variation is undoubtedly due in part to the method of measuring the bacteria in a platinum loop, although the same loop was used throughout and a sterile scalpel was used to remove all bacteria not actually in the loop. At any rate, with these

counts as a basis, an approximation of the number of bacteria in any culture can be made. In the remaining experiments, "X" is used to designate the standard suspension of bacteria. Dilutions of this standard suspension are indicated by fractions, i.e., X/4 and X/10. Suspensions of greater density are indicated by 2X, 4X, and 5X. The dilutions were made by diluting the standard suspension by the amount indicated by the fraction. The suspensions of greater density were made by adding two, four, or five times the standard amount of bacteria to 10 cc. of the balanced medium.

TABLE XII

RESULTS OF THE ISOLATION OF SINGLE ANIMALS INTO 2 DROPS OF DIFFERENT CONCENTRATIONS OF BACTERIA

	CONCENTRATION				
	4X	2X	X	X/4	X/10
Series 1	4.75	8.75	7.50	4.75	2.25
Series 2	4.00	12.25	14.50	5.00	2.25
Series 3	3.50	14.00	15.25	5.00	3.50
Series 4	2.50	8.75	10.00	3.50	2.25
Series 5	5.50	8.00	8.50	5.75	3.00
Series 6	4.00	11.50	15.75	4.00	0.50*
Series 7	2.00	5.50	8.00	6.50	2.00
Series 8	3.75	7.00	8.00	5.00	3.75
Series 9	3.50	9.50	15.25	5.50	4.25
Series 10	1.50	5.00	9.25	7.00	3.25
Series 11	1.50	6.00	10.75	7.00	4.75
Series 12	6.00	11.75	14.00	5.50	4.00
Mean	3.54	9.00	11.38	5.37	2.98

* Animals in some of the cultures died.

A set of experiments was carried out using single animals isolated into 2 drops of bacterial suspensions of 4X, 2X, X, X/4, and X/10 concentrations. The counts were made, as usual, at the end of 24 hours; the results are given in Table XII. The animals used in this and in the remaining sets of experiments were taken from pure-line cultures grown on the standard suspension of bacteria. Each number in this table is the average number of animals produced from four cultures, each starting with a single individual.

These results bring out the fact, already referred to, that there is an optimal concentration of *Pseudomonas* for reproduction in *Oxy-*

tricha. An analysis of these results shows that there is a significant difference in rate of reproduction with each increase or decrease in the concentration of bacteria from the standard suspension. The decrease in reproduction-rate in the dilute suspensions is probably due to a lack of the optimal amount of food. The cause for the decrease in the more concentrated suspensions is not so obvious. This will be taken up in the discussion.

TABLE XIII

REPRODUCTION-RATE FOR 24 HOURS FOR ONE AND TWO ANIMALS IN 2 DROPS OF 4X, 2X, AND X CONCENTRATIONS OF *Pseudomonas fluorescens*

	Concentration					
	4X		2X		X	
	1-Animal Seeding	2-Animal Seeding	1-Animal Seeding	2-Animal Seeding	1-Animal Seeding	2-Animal Seeding
Series 1.....	5.25	6.50	8.75	14.37	7.50	7.25
Series 2.....	2.75	4.50	12.25	13.00	14.50	14.00
Series 3.....	6.00	7.12	14.00	15.37	15.25	16.37
Series 4.....	6.75	7.12	8.75	10.25	9.50	8.87
Series 5.....	7.25	8.75	8.00	8.37	10.50	10.87
Series 6.....	2.75	4.12	10.50	13.75	15.75	14.75
Series 7.....	3.75	4.87	5.00	7.62	10.00	10.75
Series 8.....	3.50	4.00	7.00	8.62	8.50	9 00
Series 9.....	1.25	2.00	4.00	5.50	9.25	8.00
Series 10.....	1.50	2.12	4.00	5.12	10.75	8.37
Series 11.....	4.25	5.50	8.00	8.50	8.00	7.87
Series 12.....	5.50	5.75	8.00	11.62	8.00	8.12
Mean...	4.21	5.19	8.02	10.17	10.62	10.35

With an optimal density of bacteria for reproduction of *Oxytricha* clearly indicated, two sets of experiments were run in which animals were introduced singly and in two's in different concentrations of bacteria. The results of the use of 4X, 2X, and X concentrations are listed in Table XIII. In each series two dishes with two animals in each and four dishes with one animal in each were used for each concentration of bacteria. The numbers listed are the averages of either 2 two-animal dishes or 4 one-animal dishes at the end of 24 hours.

The results given in Table XIII show that there was a higher rate of reproduction in the two-animal cultures in both the 4X and 2X concentrations. These differences are significant. These results are

similar to those obtained in the earlier experiments using hay medium. There was no consistent difference in the rate of reproduction of the one- and two-animal cultures in the X concentration. These results seem to indicate that the density of the bacteria is a major cause of the difference in the fission-rate of one- and two-animal cultures. When densities greater than the optimum are used, it appears that two animals are able to reduce the density of the bacteria

TABLE XIV

REPRODUCTION-RATE FOR 24 HOURS FOR ONE AND FOUR ANIMALS IN 2 DROPS OF 5X AND X CONCENTRATIONS OF *Pseudomonas fluorescens*

	Concentration			
	5X		X	
	1-Animal Seeding	4-Animal Seeding	1-Animal Seeding	4-Animal Seeding
Series 1.....	2.62	3.46	8.00	5.31
Series 2.....	2.25	3.50	9.00	8.81
Series 3.....	2.75	2.62	12.00	10.68
Series 4.....	0.37*	0.93*	7.25	5.87
Series 5.....	1.25	1.53	8.00	8.00
Series 6.....	0.62*	1.50*	6.00	5.00
Mean.....	1.65	2.25	8.38	7.28

* Animals in some of the cultures died.

toward the optimum quicker than one animal, and thus are able to reproduce faster in the initial stages of the culture.

In a similar set of experiments, one- and four-animal cultures in 5X and X concentrations were studied. The results are shown in Table XIV. In each series two dishes with an initial seeding of four animals in each and eight dishes with one animal in each were used for each concentration of bacteria.

The data in Table XIII indicate that there is very little difference in the rate of division in one- and two-animal cultures in X concentrations of bacteria. Apparently, 2 drops of the standard suspension furnish adequate food for the progeny of two *Oxytricha* during 24 hours. When these results are compared with those of the X concentrations given in Table XIV, we see that the four-animal cultures, in all except one series, had a lower average reproduction-rate than the

one-animal cultures. The difference borders on significance. This seems to indicate that there is not sufficient food in X concentrations for the progeny of four *Oxytricha* during 24 hours. An examination of the cultures of 5X concentration shows that little reproduction occurred in any of the cultures. In several of the cultures some of the protozoans died. It seems obvious from this part of the study that in bacterial suspensions of such great density the protozoans are killed. The differences between the reproduction-rates in the one- and four-animal cultures of this concentration are of questionable significance.

TABLE XV

REPRODUCTION-RATE FOR 24 HOURS FOR SINGLE ANIMALS IN 2 AND 10 DROPS OF X AND X/10 CONCENTRATIONS OF *Pseudomonas fluorescens*

	Concentration			
	X		X/10	
	2 Drops	10 Drops	2 Drops	10 Drops
Series 1.....	6.50	7.50	2.00	5.25
Series 2.....	8.00	7.00	3.75	7.00
Series 3.....	8.00	8.50	5.25	7.50
Series 4.....	16.00	14.00	2.25	4.25
Series 5.....	14.00	13.50	3.50	5.00
Series 6.....	8.00	7.50	3.50	7.00
Series 7.....	13.25	12.00	0.50*	3.50
Series 8.....	9.50	9.00	3.00	7.50
Series 9.....	7.50	7.75	0.50*	1.75
Series 10.....	11.00	12.00	5.00	7.50
Mean.....	10.17	9.87	2.92	5.62

* Animals in some of the cultures died.

The last set of experiments to be reported in this paper consisted of a study of the rate of reproduction of single animals isolated into 2 and 10 drops of X and X/10 concentrations. The results given in Table XV afford some interesting comparisons. There is no significant difference in the rate of reproduction in the 2- and 10-drop cultures of X concentration. In the earlier experiments, when the nutritive hay medium was used, there was always a significant difference with this variation in volume. There is a very significant difference in the reproduction-rate of the cultures of X/10 concen-

trations: the 10-drop cultures have the higher rate. The 10-drop cultures contained five times the amount of food present in the 2-drop cultures. As 2 drops of X/10 concentration have already (Table XII) been shown to be inadequate for normal reproduction, the results here are not surprising. There is quite a difference in the results obtained in the 2-drop cultures of X concentration and in the 10-drop cultures of X/10 concentration. When the averages for these cultures are compared, one finds that the reproduction-rate of the 2-drop cultures of X concentration is much higher than that of the 10-drop cultures of X/10 concentration. The 10-drop cultures of X/10 concentration contain only half the amount of bacteria found in the 2-drop cultures of X concentration. Whether this is the only factor operating, or whether a difference in the availability of the bacteria is a factor, cannot be stated at this time.

DISCUSSION

In the first part of this work, when hay medium was used, two kinds of results were obtained, depending upon the volume of the culture medium. The rate of reproduction per animal of two *Oxytricha* seeded together was greater than that of one animal in volumes of 5 and 10 drops of medium. This was not the case in volumes of 1 and 2 drops of medium. This volume relationship was further indicated in the experiments reported in Table VI, where a decrease in rate of reproduction occurred with each increase of volume above 2 drops. These results are essentially like those found by Petersen (1929), who used *Paramecium*. This is the type of result which Robertson (1921*a* and 1921*b*) and later Yocum (1928) referred to as "allelocatalysis." As a number of workers have obtained different results in similar experiments, an attempt has been made here to further analyze the cause for these results.

The experiments reported show that the results are independent of temperature. It is shown that the results are not affected significantly by the use of paraffin-coated culture dishes. Also, variations in the hydrogen-ion concentration of the medium have not altered the type of results obtained. Of the various factors investigated, food alone offers a basis for a possible explanation of the differences discovered in the different volumes of hay medium used.

Robertson (1927) suggested that *Colpidium* and other ciliates are possibly restricted in their food to certain species of bacteria. He further suggested that the allelocatalytic effect might originate with the bacteria but that, regardless of whether it originated with the ciliates or with the bacteria, the results would be the same. In another place he suggested that the differences between his work and that of Cutler and Crump might be due to differences in the protozoan-bacteria ratio due to differences in technique. Other investigators have recognized the need of a plentiful supply of bacteria in the cultures. In most of the investigations, the presence of numerous bacteria in the cultures, introduced with the protozoan or by seeding from another culture, has been considered to be a sufficient control of the bacteria. Cutler and Crump (1924) reported that the bacteria found associated with *Colpidium* served as a better food organism than *Sarcina*, which they used. These investigators studied the effects of different numbers of bacteria on reproduction in *Colpidium* and found that an increase of bacteria caused an increase in the rate of reproduction. They did not, however, study the effects of very large numbers of bacteria, nor did they tie this up with their work on allelocatalysis. Obviously, it is impossible to analyze the results of the different investigators from the standpoint of food, when the kinds of bacteria, the suitability of the bacteria as food for the protozoan used, and the numbers of bacteria in the cultures are not known in most of the cases.

The results in the second phase of this work show that some bacteria used alone serve as suitable food for *Oxytricha*, while other kinds of bacteria used alone do not. These results are similar to those of Hargitt and Fray (1917), Phillips (1922), Philpott (1928), Losina-Losinsky (1931), and Luck, Sheets, and Thomas (1931); they seem to indicate that the presence of bacteria in large numbers in a culture does not necessarily mean that all of the bacteria are suitable food for a particular protozoan. Thus, the need of a rigid control of the kind or kinds of bacteria used in experiments on the reproductive rate in protozoans is desirable.

Not only the kinds of bacteria, but also the numbers of bacteria, are important in this work. The preliminary tests with bacterial suspensions, together with the results given in Tables XII, XIII,

XIV, and XV, show that an optimal density of bacteria is required for a good rate of reproduction in *Oxytricha*. This is not the first report of the dependence of reproduction in protozoans on an optimal density of bacteria. Chejfec (1929) found that the division-rate of *Paramecium* depends on the number of bacteria. He states that if the concentration of bacteria is too great, the division-rate is slow until an optimal number of bacteria is reached by the Infusoria eating them. The accompanying table (XVI), taken from Chejfec, illustrates his findings.

TABLE XVI

INDIVIDUAL NUMBER IN DIFFERENT CONCENTRATIONS OF *Bacillus coli* AFTER 2, 4, AND 6 DAYS (FROM CHEJFEC)

DAYS	INITIAL SEEDING	*Bacillus coli* PER CUBIC MILLIMETER				
		25,000	50,000	75,000	125,000	175,000
2	25	366	323	224	197	127
4	25	583	783	1,665	1,872	2,428
6	25	486	1,045	1,686	3,476	6,680

Barker and Taylor (1931), in using suspensions of *Pseudomonas fluorescens* as food for *Colpoda*, also found that reproduction is dependent upon the density of the bacteria. They say:

> The density of the bacterial suspension provided for the Protozoa is difficult to define except as that density in which optimal growth occurs. If too many bacteria surround the Protozoa, the latter are adversely affected as shown by a strongly reduced division rate. Definite pathologic forms may even appear. In an exceedingly dilute suspension of bacteria the Protozoa show reduction in size and division rate. In short, they behave like any undernourished organisms. Between these two extremes is a relatively wide range of bacterial suspension densities in which the division rate and the size of the *Colpoda* are maximal and approximately constant.

Some of the observations on *Drosophila* suggest a similar relationship. Eigenbrodt (1925) found that *Drosophila* grow larger in small culture vials when present in numbers of from 8 to 16 than at other population densities. Allee (1931) suggests that these observations of Eigenbrodt may be explained on the assumption "that too few *Drosophila* larvae per culture fail to control the growth of harmful

elements of the yeast or bacterial flora as well as optimal numbers do, while overcrowding overcontrols the growth of the food plant."

In the present studies, suspensions of *Pseudomonas fluorescens* in densities of 80 to 135 million per cc. (X concentration) in non-nutritive medium have been found to be optimal for reproduction in *Oxytricha*. Two drops of such suspensions (18 drops in 1 cc.) will support the progeny of either one or two *Oxytricha* for 24 hours without any significant difference in the division-rate. The fission-rate of four *Oxytricha* in the same volume of this concentration is less than that of one. In suspensions of greater density the rate of reproduction decreases with each increase in density. The use of 5X concentrations, as shown in Table XIV, almost inhibits reproduction, and in some cases causes death. Four animals in 2 drops of this suspension are unable to decrease the numbers of bacteria rapidly enough to prevent this effect.

The higher rate of reproduction found in the two-animal cultures in both 2X and 4X concentrations of bacteria is much like the type of results reported by Robertson, Yocum, and Petersen. In these experiments the cause for the results seems to be largely due to the supra-optimal densities of bacteria. As Chejfec (1929) has pointed out, when the concentration of bacteria is too great, the division-rate is slow until an optimal number of bacteria is reached by the Infusoria eating them. Two animals are able to do this faster than one, and thus, in the two-animal cultures in supra-optimal densities of bacteria, the reproduction-rate is higher than that in the one-animal cultures.

When single animals were used in 2 and 10 drops of X and X/10 concentrations, the rate of reproduction was practically the same in the cultures with X concentration of bacteria, but quite different in those of X/10 concentration. The presence of five times the amount of bacteria in 10 drops as compared with 2 drops of X/10 concentration will explain the higher reproduction in the former. As the 10-drop cultures of X/10 concentration contain only half the amount of food contained in the 2-drop cultures of X concentration, the great difference in the results may be due to the difference in the amount of the food in the two cases. However, a difference in the

availability of the bacteria suggests itself as another possible factor. More work is needed in this connection.

Although in the X/10 concentrations the 10-drop cultures had a higher rate of reproduction than the 2-drop cultures, there was almost no difference in the reproduction-rate of the 2- and 10-drop cultures of X concentration. The use of similar volumes of hay medium always gave differences in the reproduction-rate. This suggests that the rate of reproduction of *Oxytricha* for 24 hours is independent of the volume of the medium, within this range, when the density of the bacteria is optimal.

These results with suspensions of bacteria suggest that the results obtained with the hay medium may be due to the effects of the bacteria. It is taken for granted that in the hay cultures sufficient numbers of suitable bacteria were present to permit reproduction of the protozoans. In small volumes of the hay medium (1 and 2 drops) the amount of nutritive material present is much less than that in larger volumes (5, 10, 20, and 40 drops). Because of this, the larger volumes are able to support greater numbers of bacteria. In the first set of experiments it was found that 1 drop of hay medium, seeded with bacteria as described, supports the progeny of either one or two *Oxytricha* for 24 hours without any difference in the rate of reproduction. But at the end of 42 hours the one-animal cultures show a higher rate of reproduction. This seems to be due to a quicker exhaustion of the food supply in the two-animal cultures. In other experiments, when 5- and 10-drop cultures were used, the two-animal cultures had a higher rate of reproduction at the end of 24 hours. These volumes of hay medium will support greater numbers of bacteria than 1 drop will. From the data obtained, when using bacterial suspensions where the numbers of bacteria were known, it seems plausible to suggest that the effect of bacterial crowding was occurring in the larger volumes of hay medium which would account for the progressive decrease in reproduction-rate with progressive increases in volume of medium, and also for the higher reproduction-rate in the two-animal cultures of 5- and 10-drop volumes. The observation that dense clouds of bacteria existed at the end of 24 hours in the 10-, 20-, and 40-drop cultures seems to support this suggestion.

The results given in Table XIII are similar to those reported by

Robertson, Yocum, and Petersen. Those given in Table XV are similar to those reported by Woodruff, Myers, Calkins, Cutler and Crump, Greenleaf, and others. The differences in the results reported here are due to differences in the density of the bacteria in the medium. These findings suggest that the differences in the results obtained by different investigators may be due to differences in the numbers of bacteria in the cultures used. Furthermore, in considering reasons for the differences in results obtained by different investigators, the effects of unsuitable bacteria in hay infusions of unknown bacterial content should not be overlooked.

In this work no attempt has been made to prove or disprove the existence of an "X-substance" as postulated by Robertson. The results obtained here, with the bacteria controlled, show that the density of the bacteria in cultures of *Oxytricha* will explain to a considerable degree, and perhaps completely, the differences obtained in the present series of experiments upon the rate of reproduction in different population densities. Robertson reported that the interfission period was progressively shortened in the early stages of the culture. Petersen states that she did not find this to be the case. This point has not been investigated in the present work. More work is necessary in this connection, as there is some basis, on the results reported here, for supposing that protozoans in supra-optimal densities of bacteria might have their interfission periods shortened as they approach optimal densities of bacteria in the culture.

The use of conditioned medium, or medium in which an organism has lived and divided and then removed, has not been made in these experiments. The effects of toxic excretory substances, a possible "X-substance," and a decrease in the number of bacteria in the culture so that insufficient food is left, have been suggested by different investigators as having a part in the conditioning of the medium. This work indicates that other factors may enter into the conditioning, as (1) the protozoan may reduce supra-optimal numbers of bacteria to the optimum, (2) the introduced protozoan may bring into the culture more suitable bacteria, and (3) the introduced protozoan may bring into the culture harmful bacteria. That excretory substances play an important rôle in cultures of any duration is obvious. The relative rôles of food and excretory substances can be tested by

using the above mentioned technique. By using suspensions of bacteria in a given volume of non-nutritive medium, the bacteria can be replenished without changing the medium. A record of the reproduction-rate over a few days should show when excretory substances become more important than food in controlling the reproduction-rate. Experiments on this point are needed.

Further work is also needed to determine the number of bacteria consumed by a single protozoan and its progeny during the 24-hour period in different densities of bacteria. Such information should shed more light on the part played by different bacterial densities on the rate of reproduction of protozoans.

SUMMARY

1. When *Oxytricha* were isolated singly and in two's into 1 or 2 drops of hay medium, there was no difference in the rate of reproduction during 24 hours.

2. In similar experiments using animals isolated singly and in two's into 5 and 10 drops of hay medium, a higher reproduction-rate was found in the cultures starting with two animals.

3. Hay cultures, varying in volume from 1 to 40 drops and inoculated with single animals, showed the highest rate of reproduction in the 1- and 2-drop cultures and progressively decreasing rates in the 5-, 10-, 20-, and 40-drop cultures.

4. These results were found to be independent of temperature, as far as tested; of variations in the hydrogen-ion concentration of the medium within the range used; and of the use of paraffin-lined culture dishes.

5. Several of the experiments showed that increases in temperature caused increases in the reproduction-rate of the protozoans, according to usual expectations, but that relations mentioned above were not affected.

6. Suspensions of the bacterium *Pseudomonas fluorescens* in a non-nutritive balanced physiological salt medium were found to be the best type of culture medium tried for *Oxytricha*. Suspensions of *Bacillus subtilis* were less helpful, while other pure cultures of bacteria tested did not allow reproduction of *Oxytricha*.

7. Suspensions of *Pseudomonas fluorescens* in densities of 80–135

million per cubic centimeter were found to be optimal for maximal reproduction in *Oxytricha*. There was no difference in the average reproduction-rate of one and two animals for 24 hours in 2 drops of this concentration.

8. When single animals were isolated into 2 drops of medium with bacterial densities of *Pseudomonas fluorescens* two and four times greater than the optimal density, the rate of reproduction was progressively lower with each increase in density. In these supra-optimal densities, two-animal cultures had a higher reproduction-rate for 24 hours than one-animal cultures.

9. Cultures with both one and four animals, in five times the optimal density of *Pseudomonas fluorescens*, showed a marked inhibition of reproduction. In some cases the animals died in this density.

10. The rates of reproduction of single animals in 2 and 10 drops of optimal densities were about the same. The rates of reproduction of single animals in 2 and 10 drops of medium with bacterial concentrations one-tenth that of optimal concentrations were much less than those in optimal concentration, but the 10-drop cultures have the higher rate of reproduction.

11. These results show that many of the so-called allelocatalytic results obtained by Robertson can be explained on the basis of the ratio existing between the infusorian and the bacterial population.

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FACTORS WHICH MAY MODIFY PRECIPITIN TESTS IN THEIR APPLICATIONS TO ZOÖLOGY AND MEDICINE

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THE application of the precipitin reaction in fields of medicine, medico-legal work, and animal relationships is well known. It seems advisable, however, to attempt to get more reliable and shorter methods for carrying out the tests.

Uhlenhuth (1901) found that the reaction was not entirely specific but that reactions with other than the original protein might also occur. It was soon demonstrated that the degree of heterologous reactions depended upon the degree of relationship of the organisms involved. This test, then, expressed a kinship and was soon realized as another type of evidence, paralleling that from embryology or anatomy which lends support to the theory of evolution. The actual amount of evidence gathered by this biochemical method so far is meager; a great deal more of verification and extension is desirable.

From 1900 to 1904, Uhlenhuth, Wassermann and Schültze, Halban and Landsteiner, Nuttall, Linossier and Lemoine, and others carried on intensive investigation of the precipitin reaction. The results concerning the specificity of the reactions were conflicting. As late as 1920 Friedberger and Jarre reported on the non-specific activity of precipitins; yet in 1922 Manteufel and Beger found a greater specificity than most workers. The latter two publications contain many data, but the technique employed was not beyond reproach. The present opinion of immunologists is that the reaction is not entirely specific, since cross reactions occur when related animals are involved.

Nuttall in 1904 reported 16,000 qualitative tests on animal relationships and 500 (with Strangeways) quantitative tests, most of which were mammalian. Boyden in 1926 verified some of Nuttall's results, using the "ring" or "layer" method. He stressed the importance of determination of serum-protein content of the antigen and

originated the principle of reciprocal relationships as a check upon his results.

Among more recent work is that of Hayasi in 1927 on serological relationships among several whales, Moribe in 1928 on rabbit classification, and Hicks in 1929 on classification of several rodents of the *Mus* genus. Wolfe (1929), using Boyden's technique, confirmed his results and demonstrated how they might have medico-legal application. All the foregoing results correspond fairly well with known anatomical relationships.

Uhlenhuth was probably the first one to use the precipitin test for identification of blood stains in criminal cases. He established the practical importance of the test in 1902 by identifying numerous unknown stains received from "ministers of justice," medical institutes, and universities. Graham-Smith and Sanger in 1903 confirmed Uhlenhuth's results, using both qualitative and quantitative techniques.

The test has been used extensively in Europe and India to detect adulteration of foods and to identify unknown blood stains in murder, rape, and abortion cases; in the United States, however, it has been used very infrequently. Sutherland (1915–16), the imperial serologist in India, records 2,643 medico-legal cases in which 6,566 articles, suspected to be stained by blood, were examined. The evidence he procured by this scientific method aided in many convictions.

E. S. Wood (1901) was perhaps the first American to refer to the precipitin test as a possible aid in forensic medicine. (He called it the "agglutination test" and in 1902 the "serum test.") The test has been rarely used in the United States since that time. Whittier in 1902, Gay in 1908, Clarke in 1914, Hunt and Mills in 1917, and Stokes and Stoner in 1917 submitted evidence in criminal trials based upon the precipitin test. The evidence that Clarke procured convicted the person of infringement of the game laws of California; and, for the first time in this country, a person was found guilty solely on this scientific evidence.

Hektoen in 1914 and 1918 suggested using an antiserum against the whole blood, since one is often confronted with whole bloods in criminal cases. His investigations in 1922, and with Manly in 1923,

pointed out the value of the precipitin test in identification of seminal stains and, with Schulhof in 1923, showed that hemoglobin precipitins may be used in medico-legal work. The latter publication also demonstrates how selective or specific absorption may increase the specificity of the reaction.

Wolfe (1929) demonstrated a shorter method for identification of unknown blood proteins. He also used the specific absorption method for identification of the individual bloods within a mixture. One of the purposes of the present paper is to further elaborate on the methods of identification of unknown bloods with the aid of convenient serological relationship tables.

A review of the literature reveals that the precipitin reaction may be used in determination of mammalian relationships. It would be an even more convincing evidence for evolution if much more data were secured to strengthen the evidence already accumulated. The reaction is reliable and specific enough to be of medico-legal importance; and, if it is to be considered by our courts of justice, a short, precise, and accurate method of investigation of unknown stains is desirable.

The purposes of this paper are, therefore: (1) to secure additional knowledge of serological mammalian relationships, to further verify the principle of reciprocal relationships among Mammalia, and to demonstrate other methods of verification of results; (2) to illustrate how these results may be useful in shortening the procedure in identification of unknown bloods; (3) to study the effects of dilution of antiserum and of specific absorption on the specificity of the reaction and relationship values; and (4) to study the effect of age on antisera, an important item in forensic medicine.

METHODS AND MATERIAL

Healthy rabbits were used for production of precipitins. Sterile sera of beef, sheep, goat, deer, pig, horse, pony, dog, fox, cat, man, monkey, and baboon were employed for injections. The usual and more successful method of injection was by the intravenous route. Under aseptic conditions five injections were made with increasing dosage of 1–5 cc. at intervals of 3 or 4 days. Intraperitoneal injections were also made, inasmuch as several casualties resulted from

the former method. The results secured by the intraperitoneal method were not as satisfactory as those by the intravenous: the titers were lower, and larger amounts of antigen were required. Typical anaphylactic shock resulted several times after the third or fourth injection. The only explanation seems to be the great individual variation among the animals used.

Eight days after the last injection a preliminary titration was made to determine the potency of the antiserum. If the titer by ring test was 1:12,800 or above, the rabbit, after being anesthetized, was bled completely from the heart on the next day after 12 hours or more of starvation. Both antigen and antiserum were kept in ampules after sterilization by filtration through Seitz filters.

The ring test was used throughout the experiments. Two per cent standard antigen solutions were prepared from the above mentioned sera. The following blood sera were also used as "test antigens": llama, badger, skunk, panther, chimpanzee, prong buck, fallow deer, rat, guinea pig, and rabbit. In carrying out dilutions and calculating relationship and reciprocal relationship values, the method used by Boyden was used exclusively. This method consisted of making serial dilutions of the standard antigen with buffered salt solution. The highest dilution in which a reaction occurred was considered 100 per cent, and the heterologous reactions were recorded in percentages of the homologous titers.

Protein determinations were made on the 2 per cent standard solutions. All readings of the tests were made at a temperature of $37.5^{\circ} \pm 1$ at intervals of 20, 40, and 60 minutes. Final results were based on the 60-minute reading. Most tests were carried out in duplicate, some in triplicate, and about twenty of the some thousand tests were carried out singly because of the lack of antiserum. In most cases two and three antisera against the same species were used to check results.

The sera were secured from several sources. The blood of the more common domesticated animals was obtained from the University of Wisconsin College of Agriculture and from the Oscar Mayer Packing Company. Badger and fox bloods were secured from farmers, deer blood from the Madison zoo, and the chimpanzee and baboon blood from Yale University through Dr. Fulton of the physiolo-

gy department. The other samples of blood, consisting of whole blood preserved in thymol and obtained from autopsies, were secured from the Milwaukee zoo through the courtesy of Professor Swindle of Marquette University.

EXPERIMENTAL RESULTS

1. *Antisera, protein content of antigen, and range of error.*—Table I shows the antisera used in the experiments. The results and other data on hand indicate that the intravenous route of injection is better as the amount of antigen required is less; and, secondly, that the titer is as high, if not higher, than that resulting from the intraperitoneal route. The results are much more consistent when the former method is used. When more than one antiserum was produced against a species, sera of different animals were employed; but the test antigen was always of only one of the animals. This, it was believed, would make the results more comparable. Three of the antisera showed a titer of only 1:6,400, though a preliminary titration indicated a titer of 1:12,800. The effect of titer on relationships and specificity can also be derived from this table and the work which follows.

Table II gives the protein concentration of the standard solutions used throughout the work. Folin and Wright's simplified macro-Kjeldahl method (1919) was used for determination of total nitrogen. Protein was calculated as total nitrogen $\times$ 6.25. This would give figures somewhat too high, on account of small amounts of non-protein nitrogen present in the sera. The difference would not be significant, considering the error of the ring test itself. The percentage of protein in the sera of the various species agrees very well with those of other workers.

The advisability of determining protein concentration can be seen from the table, for there is variation of protein content even within a species. With the "test antigens" used throughout this work, relationship value corrections were not necessary. Table III demonstrates that corrections for protein would not be significant in this relationship data. In medico-legal work, on the other hand, protein determinations are unmistakably necessary.

The greatest deviation in the reading test was plus or minus one

TABLE I

ANTISERA USED IN THE FOREGOING EXPERIMENTS

Antiserum Sample No.	No. of Serum Used for Injection	No. of Test Antigen	Cubic Centimeters Injected	Method of Injection*	Date of Preparation	Titer
Beef:						
♂MII	11	11	10	Intravenous	2-5-30	1:12,800
♀UV12	12	11	10	Intravenous	12-22-30	1:12,800
♂C11	11	11	14.5	Intravenous	4-22-30	1:12,800
Sheep:						
♀P20	20	21	15	Intravenous	2-8-30	1:25,600
♂P21	21	21	14.5	Intravenous	3-9-30	1:12,800
Goat:						
♀S30	30	30	10	Intravenous	3-26-30	1:12,800
♀HW4	33	30	15	Intravenous	7-15-31	1:25,600
♀H35	32	30	21.5	Both	2-3-32	1:6,400
Deer (white-tailed):						
♀PHW15	210	210	42.5	Intraperitoneal	11-12-31	1:25,600
Pig:						
♂T36	36	36	15	Intravenous	4-22-30	1:12,800
♂HW39	36	36	30	Intraperitoneal	10-20-31	1:12,800
Horse:						
♂K2b	2	1	30	Intravenous	6-24-30	1:6,400
♂H20	1	1	18.5	Both	1-27-32	1:12,800
Pony:						
♀HW31	50	50	14	Both	11-5-31	1:12,800
Dog:						
♀PN15C17	17	17	21	Intravenous	1-22-31	1:12,800
♀N15	15	17	15	Intravenous	2-8-30	1:12,800
Fox:						
♀H33	216	216	23.5	Both	2-6-32	1:12,800
Cat:						
♀R25c	25	27	15	Intravenous	3-25-30	1:6,400
♀HW29	27	27	23.5	Both	11-17-31	1:12,800
Baboon:						
♀H36	305	305	21.5	Both	2-3-32	1:25,600
Monkey:†						
♀L5	5	5	15	Intravenous	2-7-30	1:25,600
♂L6	6	5	21	Intravenous	10-22-30	1:25,600
Man:						
♀H205	205	205	30	Both	2-6-32	1:25,600

* Intravenously or intraperitoneally or both.

† *Macacus rhesus.*

tube, or a possible error of 100 per cent. This error is not high when we compare the sensitivity of the precipitin test in the detection of protein with any other means known. Moreover, of the some thou-

TABLE II

PROTEIN DETERMINATIONS ON STANDARD (2 PER CENT) ANTIGEN SOLUTIONS

Serum Sample No.	Date of Preparation	Date of Determination	Total N. per 100 Cc. of Serum	Protein in Grams per 100 Cc. of Serum
Man 205	11-6-31	2-4-32	1.299	8.12
Man 206	2-4-32	2-4-32	1.324	8.277
Monkey 5	7-1-31	2-4-32	0.957	6.09
Monkey 7	2-3-32	2-3-32	1.043	6.523
Baboon 305	12-11-31	2-4-32	1.079	6.746
Chimpanzee 405	1-4-32	2-2-32	1.217	7.612
Lemur* 105	10-7-31	2-3-32	1.938	12.118
Beef 11	7-2-31	12-23-31	1.26	7.875
Beef 12	2-3-32	2-3-32	1.043	6.518
Sheep 21	7-14-31	12-23-31	1.374	8.592
Sheep 22	7-14-31	2-2-32	1.165	7.284
Goat 30	7-2-31	2-1-32	1.344	8.40
Goat 33	2-4-32	2-4-32	1.107	6.921
Deer 210 (white-tailed)	11-14-31	2-4-32	1.114	6.965
Deer 310 (fallow)*	11-14-31	2-2-32	0.863	5.398
Pig 35	2-4-32	2-4-32	1.201	7.511
Pig 36	7-14-31	2-1-32	1.309	8.181
Llama 55	6-23-31	2-23-31	1.134	7.087
Pecarry* 135	7-1-31	2-2-32	1.113	6.956
Horse 1	7-1-31	2-1-32	1.371	8.574
Horse 2	2-3-32	2-3-32	1.227	7.673
Pony 50	6-23-31	12-23-31	1.085	6.781
Dog 17	6-24-31	2-1-32	1.162	7.262
Dog 18	7-1-31	2-2-32	1.015	6.343
Cat 25	2-3-32	2-3-32	1.141	7.135
Cat 27	6-23-31	2-1-32	1.221	7.634
Badger 75	6-23-31	2-1-32	0.948	5.928
Fox 216	11-17-31	2-3-32	0.974	6.09
Skunk* 125	7-1-31	2-2-32	1.079	6.146
Panther* 115	7-1-31	2-2-32	1.575	9.843
Rat 40	7-14-31	2-2-32	0.745	4.659
Rabbit 80	7-21-31	2-1-32	1.33	8.312
Guinea pig 90	7-21-31	2-2-32	0.811	5.074

* Whole blood, preserved in thymol.

sand duplicate tests which were run, less than 1 per cent showed a deviation of plus or minus one tube, while the other readings were exactly the same.

2. *Relationships.*—Table IV shows the relationships of three individual anti-beef sera against ungulates, carnivores, and primates. The high heterologous titers of the anti-beef serum with the goat,

TABLE III

COMPARISON OF RELATIONSHIPS AS BASED ON PROTEIN CORRECTION

Antigen	Two Anti-horse Sera			
	♂K2b		♂H20	
	Actual Reading (Percentage)	Corrected for Protein Content (Percentage)	Actual Reading (Percentage)	Corrected for Protein Content (Percentage)
Horse	100	100	100	100
Pony	150	179	150	179
Beef	18.75	20.29	9.37	10.14
Sheep	18.75	18.69	6.25	6.23
Goat	18.75	19.12	6.25	6.375
Deer	6.25	8.68	3.12	4.34
Pig	25	28.5	12.5	14.25
Llama	12.5	16	3.12	4
Dog	6.25	7.37	3.12	3.585
Fox	6.25	8.75	3.12	4.375
Cat	9.38	10.53	3.12	3.5
Badger	12.5	18	6.25	9
Baboon	9.38	12.01	6.25	7.93
Monkey	12.5	17.5	6.25	8.75
Chimpanzee	12.5	14.12	6.25	7.062
Man	9.38	9.89	4.68	4.92

TABLE IV

RELATIONSHIPS

Antigen	Three Anti-beef Sera		
	♀ UV12 (Percentage)	♂C11 (Percentage)	♂M11 (Percentage)
Beef	100	100	100
Sheep	50	50	50
Goat	50	50	50
Deer (white-tailed)	50	25	.....
Deer (fallow)*	3.12	3.12	.....
Pig	3.12	12.5	6.25
Llama	2.3	3.12	1.56
Prong buck*	12.5	.....	.....
Horse	6.25	4.7	6.25
Pony	6.25	3.12	6.25
Dog	1.56	1.56	3.12
Fox	3.12	1.56	.....
Cat	1.56	1.56	6.25
Badger	3.12	1.56	.....
Man	3.12	1.56	.....
Chimpanzee	4.67	3.12	.....
Monkey	6.25	3.12	.....
Baboon	6.25	1.56	.....

* Whole blood.

sheep, and deer antigens reveals a very close relationship of these four animals. The fallow-deer reactions were very weak and may have resulted from the presence of thymol. Moreover, the protein used as a test antigen was whole blood and may have been low in serum protein content. A comparison of the values of the three anti-beef sera discloses a very close agreement with the exception of the pig and cat antigens.

Table V records the results of three anti-goat sera. As in the previous table, there is a close kinship noticed among goat, sheep, beef,

TABLE V

RELATIONSHIPS

Antigen	Three Anti-goat Sera		
	♀ S30 (Percentage)	♀ HW4 (Percentage)	♀ H35 (Percentage)
Goat	100	100	100
Sheep	100	75	50
Beef	50	50	50
Deer (white-tailed)	52	25	25
Deer (fallow)	.....	1.56	3.12
Pig	6.25	12.5	9.4
Llama	3.12	12.5	6.25
Prong buck*	12.5	.....	.....
Horse	6.25	12.5	6.25
Pony	6.25	12.5	6.25
Dog	1.56	3.12	6.25
Fox	.....	4.6	.....
Cat	1.56	3.12	0
Badger	2.3	2.3	6.25
Man	2.3	12.5	3.12
Chimpanzee	3.12	18.75	6.25
Monkey	4.6	12.5	6.25
Baboon	3.12	6.25	6.25
Rabbit	.....	0	.....

* Whole blood.

and deer. Goat seems to be more closely related to sheep than to beef or deer. The ♀ S30 and ♀ H35 results coincide very closely within the range of error of the test. The values of ♀ HW4 are slightly higher than the other two. Boor and Hektoen (1930) and Hektoen and Boor (1931), using hemoglobin as an antigen, found that a greater extra-specific action may be obtained by extra immunization. The foregoing results, and other data which will be reported subsequently, indicate that the cause of this extra-specificity is due to the varia-

tion of response of the antibody producers—the rabbits. At times an antiserum produced by a single series of injections gave the higher heterologous reactions; and at other times, the lower. These results are not strictly comparable to Hektoen and Boor's, as they used the sera of a single rabbit after the first and second series of injections, while the foregoing conclusions were derived from different rabbits.

TABLE VI

RELATIONSHIPS

ANTIGEN	TWO ANTI-SHEEP SERA	
	♀ P20 (Percentage)	♂P21 (Percentage)
Sheep	100	100
Goat	100	75
Beef	50	50
Deer (white-tailed)	75	50
Deer (fallow)*	6.25	4.67
Pig	6.25	6.25
Llama	1.56	3.12
Prong buck*	6.25	12.5
Horse	2.3	12.5
Pony	3.12	12.5
Dog	1.56	4.67
Fox	1.56	1.56
Cat	1.56	3.12
Badger	2.3	3.12
Man	1.56	3.12
Chimpanzee	3.12	6.25
Monkey	3.12	6.25
Baboon	2.3	3.12
Guinea pig	0.8	1.6
Rat	.....	0.4

* Whole blood.

Table VI represents the reactions of two anti-sheep sera; and Table VII, of an anti-deer serum. Once more the close relationship of deer, beef, goat, and sheep is verified, the latter two again indicating the closest relationship. The values for ♂P21 are higher than the values for ♀ P20 in several instances, though both received but a single series of injections.

Rabbit ♀ P20 was injected with serum which was 3 years old, but the antigenicity of the blood was not altered in the production of a high homologous titer. An antiserum produced 3 years previously against the same serum yielded much higher cross reactions with

horse, pig, and human sera. Since injections of the fresher serum resulted in higher heterologous tests, is it possible that its age may affect the power of an antigen quantitatively in its production of heterologous reactions? Or were these results due to the variation of the rabbits? The former inference seems doubtful, though further work in this field is necessary.

TABLE VII

RELATIONSHIPS

ANTIGEN	ANTI-DEER SERUM ♀ PHW15 (Percentage)
Deer (white-tailed)	100
Beef	50
Goat	50
Sheep	50
Deer (fallow)*	12.5
Llama	1.56
Pig	12.5
Horse	3.12
Pony	4.7
Dog	1.17
Fox	0.59
Cat	0.78
Badger	1.56
Man	1.56
Monkey	2.33
Baboon	2.33

* Whole blood.

Two series of tests were made against ♀ PHW15. In one series the antiserum was not diluted, and in the second series it was diluted 1:1 with buffered salt solution. The titers were the same, but the readings were more distinct in the case of the diluted antiserum. These results are of especial importance and will be further elaborated upon.

Tables VIII, IX, and X list the relationship values secured against five Carnivora antisera representing three species. The results of the two anti-cat series bring out the close kinship of the panther and cat. ♀ HW29 reactions yielded higher reactions with members of the Carnivora group than other animals, while ♀ R25c did not. The former received two series of injections; the latter, but one. Fox and

dog show an expected very close resemblance. The parallel series are comparable within the range of error.

Table XI represents the blood tests of two anti-horse sera and one anti-pony serum. The similarity of results of the two animals is expected. ♂K2b, alone of the three sera, was produced by two series of injections; and it yielded the higher heterologous reactions. On

TABLE VIII

RELATIONSHIPS

ANTIGEN	TWO ANTI-CAT SERA	
	♀ R25c (Percentage)	♀ HW29 (Percentage)
Cat	100	100
Panther*	25	50
Dog	6.25	25
Fox (silver)	6.25	18.75
Badger	12.5	12.5
Skunk*	.....	25
Beef	9.4	9.4
Goat	6.25	6.25
Sheep	9.4	6.25
Deer	.....	3.12
Pig	12.5	25
Llama	6.25	3.12
Horse	12.5	9.4
Pony	12.5	6.25
Man	12.5	9.4
Monkey	4.7	6.25
Baboon	.....	6.25
Guinea pig	0	0
Rat	.....	0
Rabbit	0	.....

* Whole blood.

the other hand, an antiserum ♂K1 produced by a single series of injections gave even higher cross reactions. (This series is not reported because of lack of antiserum.) This again seems to illustrate the previous statement that the degree of heterologous reactions depends in part upon the animal injected.

The anti-horse serum with pony blood gave higher values than the homologous test, though within the limits of error.

The two series of anti-pig serum reactions agree very closely. In most cases the relationship values against hoofed forms are higher than against other animals. This indicates a slightly closer kinship

with these forms. From these and reciprocal results it can be inferred that the pig is rather distantly related to the goat, cow, sheep, and horse. These forms, though originating on the "ungulate stem of the tree," seem to have diverged quite considerably.

TABLE IX

RELATIONSHIPS

Antigen	Two Anti-dog Sera	
	♀ N15 (Percentage)	♀ PN15C17 (Percentage)
Dog	100	100
Fox (silver)	100	100
Cat	6.25	12.5
Badger	6.25	12.5
Panther*	6.25	6.25
Skunk*	6.25	6.25
Beef	6.25	9.3
Goat	6.25	6.25
Sheep	6.25	6.25
Deer	3.12	.....
Pig	3.12	12.5
Llama	3.12	6.25
Horse	6.25	12.5
Pony	6.25	12.5
Man	3.12	3.12
Chimpanzee	4.7	3.12
Macacus	3.12	2.33
Baboon	6.25	6.25
Guinea pig	1.56	.....
Rat	0	.....
Rabbit	0	.....

* Whole blood.

Tables XIII, XIV, and XV contain the relationships of four anti-primate sera of three different species. In each case, with the exception of the lemur, a definite group reaction resulted. Table XIII shows that baboon is very closely related to *Macacus*. The tests of the two anti-*Macacus* sera indicate that there is a close relationship among the four primates, and the lemur is very distantly related. The baboon, indeed, appears to be a closer relation to *Macacus* than to chimpanzee or man. These facts verify our knowledge of anatomical relationships. It is clear that human blood serum could be easily distinguished from sera of animals below that of the higher primates.

TABLE X

RELATIONSHIPS

ANTIGEN	ANTI-FOX SERUM
	♀ H_{33} (Percentage)
Fox (silver)	100
Dog	75
Cat	12.5
Badger	12.5
Panther*	6.25
Skunk*	9.4
Beef	9.4
Sheep	12.5
Goat	6.25
Deer	0.78
Pig	12.5
Llampaca	3.12
Horse	12.5
Pony	3.12
Man	3.12
Chimpanzee	6.25
Monkey	3.12
Baboon	6.25
Rat	0
Guinea pig	0.78

* Whole blood.

TABLE XI

RELATIONSHIPS

ANTIGEN	TWO ANTI-HORSE SERA		ANTI-PONY SERUM
	♂ H_{20} (Percentage)	♂ K_{2b} (Percentage)	♀ HW_{31} (Percentage)
Horse	100	100	100
Pony	150	150	100
Beef	9.4	18.7	6.25
Sheep	6.25	18.7	6.25
Goat	6.25	18.7	6.25
Deer	3.12	6.25	3.12
Pig	12.5	25	6.25
Llama	3.12	12.5	1.56
Fox	4.7	6.25	3.12
Dog	3.12	6.25	3.12
Cat	3.12	9.4	3.12
Badger	6.25	12.5	6.25
Man	2.35	4.7	4.7
Chimpanzee	6.25	12.5	6.25
Monkey	6.25	12.5	3.12
Baboon	6.25	9.4	6.25
Rat	0	0	.....
Guinea pig	0.78	6.25	.....

TABLE XII

RELATIONSHIPS

ANTIGEN	TWO ANTI-PIG SERA	
	♂T36 (Percentage)	♂HW39 (Percentage)
Pig	100	100
Beef	6.25	9.4
Sheep	6.25	12.5
Goat	6.25	6.25
Deer	3.12	4.67
Peccary*	.....	0.4
Llama	3.12	3.12
Horse	6.25	9.4
Pony	12.5	12.5
Dog	1.56	3.12
Fox	1.56	1.56
Cat	1.56	3.12
Badger	4.67	3.12
Man	2.33	3.12
Chimpanzee	3.12	9.4
Monkey	4.67	4.67
Baboon	3.12	6.25
Rat	0	.....

* Whole blood.

TABLE XIII

RELATIONSHIPS

ANTIGEN	ANTI-BABOON SERUM
	♀H36 (Percentage)
Baboon	100
Monkey	50
Chimpanzee	12.5
Man	25
Lemur*	0.78
Beef	4.7
Sheep	3.12
Goat	6.25
Deer	1.56
Pig	3.12
Llama	1.56
Horse	6.25
Dog	1.56
Fox	2.3
Cat	2.3
Badger	2.3

* Whole blood.

TABLE XIV

RELATIONSHIPS

ANTIGEN	TWO ANTI-*Macacus* SERA	
	♀ L_5 (Percentage)	♂ L6 (Percentage)
Macacus	100	100
Man	100	150
Chimpanzee	100	50
Baboon	100	100
Lemur*	4.7	9.4
Beef	3.12	4.72
Sheep	3.12	3.12
Goat	4.7	3.12
Deer	0.78	3.12
Pig	3.12	4.7
Llama	0.78	1.2
Horse	3.12	3.12
Pony	3.12	3.12
Dog	0.78	0.78
Fox	.....	.....
Cat	1.56	0.78
Badger	1.56	0.78
Guinea pig	.4	0.6
Rat	0	.....
Rabbit	0	.....

* Whole blood.

TABLE XV

RELATIONSHIPS

ANTIGEN	ANTI-HUMAN SERUM
	♀ H_{205} (Percentage)
Man	100
Champanzee	75
Monkey	50
Baboon	50
Lemur*	1.17
Beef	3.12
Sheep	1.56
Goat	3.12
Deer	2.33
Pig	2.33
Horse	3.12
Pony	12.5
Llama	2.33
Dog	2.33
Fox	2.33
Cat	1.17
Badger	3.12

* Whole blood.

TABLE XVI

RECIPROCAL RELATIONSHIPS

Antiserum	Antigen	Percentage of Relationship	Antiserum	Antigen	Percentage of Relationship
Anti-beef	Sheep	50	Anti-sheep	Beef	50
	Goat	50	Anti-goat	Beef	50
	Deer	50	Anti-deer	Beef	50
	Pig	3.12	Anti-pig	Beef	6.25
	Horse	6.25	Anti-horse	Beef	6.25
	Pony	6.25	Anti-pony	Beef	6.25
	Fox	3.12	Anti-fox	Beef	9.4 *
	Dog	1.56	Anti-dog	Beef	6.25*
	Cat	1.56	Anti-cat	Beef	9.4 *
	Man	3.12	Anti-man	Beef	3.12
	Macacus	6.25	Anti-*Macacus*	Beef	3.12
	Baboon	6.25	Anti-baboon	Beef	3.12
Anti-sheep	Goat	100	Anti-goat	Sheep	100
	Deer	75	Anti-deer	Sheep	50
	Pig	6.25	Anti-pig	Sheep	6.25
	Horse	2.3	Anti-horse	Sheep	6.25*
	Pony	3.12	Anti-pony	Sheep	6.25
	Dog	1.56	Anti-dog	Sheep	6.25*
	Fox	1.56	Anti-fox	Sheep	12.5 *
	Cat	1.56	Anti-cat	Sheep	9.4 *
	Man	1.56	Anti-man	Sheep	1.56
	Macacus	3.12	Anti-*Macacus*	Sheep	3.12
	Baboon	2.3	Anti-baboon	Sheep	3.12
Anti-goat	Deer	25	Anti-deer	Goat	50
	Pig	6.25	Anti-pig	Goat	6.25
	Horse	6.25	Anti-horse	Goat	6.25
	Pony	6.25	Anti-pony	Goat	6.25
	Dog	1.56	Anti-dog	Goat	6.25*
	Fox	4.6	Anti-fox	Goat	6.25
	Cat	1.56	Anti-cat	Goat	6.25*
	Man	2.3	Anti-man	Goat	3.12
	Macacus	4.7	Anti-*Macacus*	Goat	4.7
	Baboon	3.12	Anti-baboon	Goat	6.25
Anti-deer	Pig	12.5	Anti-pig	Deer	3.12*
	Horse	3.12	Anti-horse	Deer	3.12
	Pony	4.7	Anti-pony	Deer	3.12
	Dog	1.17	Anti-dog	Deer	3.12*
	Fox	0.59	Anti-fox	Deer	0.78
	Cat	0.78	Anti-cat	Deer	3.12*
	Man	1.56	Anti-man	Deer	2.33
	Macacus	2.33	Anti-*Macacus*	Deer	0.78*
	Baboon	2.33	Anti-baboon	Deer	1.56
Anti-pig	Horse	6.25	Anti-horse	Pig	12.5
	Pony	12.5	Anti-pony	Pig	6.25
	Dog	0.78	Anti-dog	Pig	3.12*
	Fox	1.56	Anti-fox	Pig	12.5 *

* Outside limit of error of test.

TABLE XVI—*Continued*

Antiserum	Antigen	Percentage of Relationship	Antiserum	Antigen	Percentage of Relationship
Anti-pig—*Continued*					
	Cat	1.56	Anti-cat	Pig	12.5 *
	Man	1.17	Anti-man	Pig	2.33
	Macacus	2.33	Anti-*Macacus*	Pig	3.12
	Baboon	3.12	Anti-baboon	Pig	3.12
Anti-horse	Pony	150	Anti-pony	Horse	100
	Dog	3.12	Anti-dog	Horse	6.25
	Fox	4.7	Anti-fox	Horse	12.5 *
	Cat	3.12	Anti-cat	Horse	12.5 *
	Man	2.33	Anti-man	Horse	3.12
	Macacus	6.25	Anti-*Macacus*	Horse	3.12
	Baboon	6.25	Anti-baboon	Horse	6.25
Anti-pony	Dog	3.12	Anti-dog	Pony	6.25
	Fox	3.12	Anti-fox	Pony	3.12
	Cat	3.12	Anti-cat	Pony	6.25
	Man	4.7	Anti-man	Pony	12.5 *
	Macacus	3.12	Anti-*Macacus*	Pony	3.12
Anti-dog	Fox	75	Anti-fox	Dog	100
	Cat	12.5	Anti-cat	Dog	6.25
	Man	3.12	Anti-man	Dog	2.33
	Macacus	3.12	Anti-*Macacus*	Dog	0.78*
	Baboon	6.25	Anti-baboon	Dog	1.56*
Anti-fox	Cat	6.25	Anti-cat	Fox	6.25
	Man	3.12	Anti-man	Fox	2.33
	Baboon	6.25	Anti-baboon	Fox	2.3 *
Anti-cat	Man	12.5	Anti-man	Cat	1.17*
	Baboon	6.25	Anti-baboon	Cat	2.3 *
Anti-*Macacus*	Man	100	Anti-man	*Macacus*	50
	Baboon	100	Anti-baboon	*Macacus*	50
Anti-man	Baboon	50	Anti-baboon	Man	25

To test the accuracy and reliability of the results, Boyden advanced the principle of reciprocal relationships. Table XVI gives the values obtained in 75 reciprocal titrations. The data were taken at random, using the first series of reactions from the preceding tables.

Twenty-four of the 75 values do not agree within the limits of error of the test. A very significant point is that 20 of these 24 involve reactions of the carnivores. In every one of these 20 cases the anti-Carnivora serum gave higher values than the reciprocals. What is the cause of this? Dr. Guyer, of this department, has suggested

that it may be due to the diet of the animals. This is very probable, and blood of animals starved for a short time and then used for antibody production may clear up this discrepancy.

Another means of verification of results seems possible. It is logical to assume that very closely related species should show the same degree of relationship to more distantly related forms. Table XVII is a comparison of four Ungulata antisera. The results check each other very nicely. In Table XI the anti-pony and anti-horse compare very favorably, as would a comparison of the primate series.

TABLE XVII

RELATIONSHIPS—COMPARATIVE

ANTIGEN	FOUR ANTI-UNGULATA SERA			
	Beef (Percentage)	Sheep (Percentage)	Goat (Percentage)	Deer (Percentage)
Beef	100	50	50	50
Sheep	50	100	100	50
Goat	50	100	100	50
Deer (white-tailed)	50	75	25	100
Pig	3.12	6.25	6.25	12.5
Llama	2.3	1.56	3.12	1.56
Deer (fallow)	3.12	6.25	1.56*	12.5
Prong buck	12.5	6.25	12.5	.....
Horse	6.25	2.3	6.25	3.12
Pony	6.25	3.12	6.25	4.7
Dog	1.56	1.56	1.56	1.17
Fox	3.12	1.56	4.6 *	0.59
Cat	1.56	1.56	1.56	0.78
Badger	3.12	2.3	2.3	1.56
Man	3.12	1.56	2.3	1.56
Chimpanzee	4.67	3.12	3.12	.....
Monkey	6.25	3.12	4.6	2.33
Baboon	6.25	2.3	3.12	2.33

* Antisera ♀ HW_4.

The dog and fox appear rather distantly related to the cat. In the evolution of these forms it seems plausible that they diverged equally from a certain point after a common development on the carnivore branch of the "phylogenetic tree." Table XVIII demonstrates that this type of comparison is feasible if the assumption established is correct.

A comparison of some of the results obtained with those of Nuttall and Boyden is given in Table XIX. The table records the tests of an anti-pig and an anti-sheep serum. The general correspondence of

the results is obvious, and the value of this biological method for the study of the problem of relationships is, without doubt, trustworthy.

3. *Effect of dilution of antisera on specificity and relationship values.*—Boor and Hektoen (1930) and Hektoen and Boor (1931) found that anti-hemoglobin and anti-carbonmonoxide hemoglobin sera reactions could be made to lose most of their extra-specific reactions if

TABLE XVIII

RELATIONSHIPS—COMPARATIVE

ANTIGEN	THREE ANTI-CARNIVORA SERA		
	Dog ♀ N15 (Percentage)	Fox ♀ H33 (Percentage)	Cat ♀ R25c (Percentage)
Dog	100	75	6.25
Fox	100	100	6.25
Cat	6.25	12.5	100
Badger	6.25	12.5	12.5
Panther	6.25	6.25	25
Skunk	6.25	9.4	25*
Beef	6.25	9.4	9.4
Sheep	6.25	12.5	9.4
Goat	6.25	6.25	6.25
Deer	3.12	0.78	3.12*
Pig	3.12	12.5	12.5
Llama	3.12	3.12	6.25
Horse	6.25	12.5	12.5
Pony	6.25	3.12	12.5
Man	3.12	3.12	12.5
Chimpanzee	4.7	6.25	.....
Monkey	3.12	3.12	4.7
Baboon	6.25	6.25	6.25*
Guinea pig	1.56	0.78	0
Rat	0	0	0*

* Antisera ♀ HW29.

the precipitin solution was diluted 1:3 with saline solution or normal rabbit serum. This dilution removed all heterologous reactions with the exception of those of very closely related animals, such as beef and yak or *Macacus* and man. An antiserum could be produced which would remove cross tests between an anti-beef hemoglobin serum and sheep hemoglobin.

Hemoglobin immunological reactions seem to show in general a greater species specificity (Hektoen and Shulhof, 1923) than serum proteins. This lack of extra-specific reaction is, of course, a handicap

in relationship work. Since dilution of a hemoglobin precipitin solution eliminates most cross reactions, it would be interesting to know whether the same holds true for serum precipitins and the effect that this dilution may have on relationship values.

Two sets of experiments were made: one series involved an anti-goat serum; the other, an anti-beef serum. The titer of the former

TABLE XIX

COMPARISON OF RELATIONSHIPS

Antiserum	Antigen	Nuttall and Strangeways* (Percentage)	Boyden (Percentage)	Wolfe (Percentage)
Anti-sheep	Sheep	100	100	100
	Goat	39	75	100
	Beef	37	50	50
	Pig	4	3.1	6.25
	Horse	3	3.1	2.3
	Cat	4	.....	1.56
	Dog	7	3.1	1.56
	Man	1.5	0.58	1.56
	Baboon	4	.....	2.3
	Guinea pig	0	.....	0.8
Anti-pig	Pig	100	100	100
	Horse	12	9.4	6.25
	Goat	9	6	6.25
	Beef	7	7	6.25
	Sheep	7	6	6.25
	Man	6	3.1	2.33
	Dog	6	4.7	1.56
	Cat	9	.....	1.56

* *Blood Immunity and Relationships.*

was 1:6,400; of the latter, 1:25,600. Dilutions of antisera with buffered saline were made ranging from a 1:1 to 1:10. Test antigens used were those of animals which gave definite group reactions and also of more distantly related forms, such as pig, horse, *Macacus*, and fox or dog.

Table XX records the anti-beef series and demonstrates the effect on specificity. A dilution of 1:2 is practically without effect, while 1:3 diminishes only two cross reactions of the extra-group blood. The 1:5 and 1:6 antisera show weaker heterologous reactions throughout, but far from absolute specificity. The 1:10 solution gave considerably weaker reactions, but only the test against fox serum was negative.

Table XXI reveals that antiserum dilutions as high as 1:4, inclusive, have but little effect on relationship values. Dilutions beyond that, especially the 1:10 serum, have much greater effect.

TABLE XX

EFFECT OF DILUTION OF ANTISERUM ON TITER

ANTIGEN	ANTI-BEEF SERUM: ♂C11						
	No Dilution	1:2 Dilution	1:3 Dilution	1:4 Dilution	1:5 Dilution	1:6 Dilution	1:10 Dilution
Beef	1:25,600	1:25,600	1:25,600	1:25,600	1:25,600	1:25,600	1:12,800
Goat	1:12,800	1:12,800	1:12,800	1:12,800	1:6,400	1:6,400	1:3,200
Sheep	1:12,800	1:12,800	1:12,800	1:6,400	1:6,400	1:6,400	1:2,400 av.
Deer (white-tailed)	1:6,400	1:6,400	1:4,800	1:3,200	1:3,200	1:3,200	1:800
Pig	1:3,200	1:2,400 av.	1:1,600	1:1,600	1:1,800	1:1,800	1:200
Horse	1:1,200 av.	1:800	1:800	1:800	.....	1:400	1:200
Monkey	1:800	1:800	1:800	1:800	1:400	1:400	1:400
Fox	1:400	1:400	1:200	1:200	1:200	1:100	0

TABLE XXI

EFFECT OF DILUTION OF ANTISERUM ON RELATIONSHIP VALUES

ANTIGEN	ANTI-BEEF SERUM: ♂C11						
	No Dilution (Percentage)	1:2 Dilution (Percentage)	1:3 Dilution (Percentage)	1:4 Dilution (Percentage)	1:5 Dilution (Percentage)	1:6 Dilution (Percentage)	1:10 Dilution (Percentage)
Beef	100	100	100	100	100	100	100
Goat	50	50	50	50	25	25	25
Sheep	50	50	50	25	25	25	18.75
Deer	25	25	18.75	12.5	12.5	12.5	6.25
Pig	12.5	9.4	6.25	6.25	3.12	3.12	1.56
Horse	4.7	3.12	3.12	3.12	.....	1.56	1.56
Monkey	3.12	3.12	3.12	3.12	1.56	1.56	3.12
Fox	1.56	1.56	0.78	0.78	0.78	0.39	0

Tables XXII and XXIII contain the results for antiserum ♀H35. Here again a dilution of 1:2 was without effect, but the higher ones yielded lower heterologous reactions. The tests of the 1:10 antiserum were positive only for the "group" forms. Relationship values were altered by the 1:4 antiserum and considerably so by sera of still higher dilutions.

These results indicate that serum precipitins are not affected as readily as are hemoglobin precipitins by addition of saline. The difference in reactions between these two substances is one of degree

TABLE XXII

EFFECT OF DILUTION OF ANTISERUM ON TITER

ANTIGEN	ANTI-GOAT SERUM: ♀ H35					
	1:1 No Dilution	1:2 Dilution	1:4 Dilution	1:6 Dilution	1:8 Dilution	1:10 Dilution
Goat	1:6,400	1:6,400	1:6,400	1:6,400	1:6,400	1:4,800 av.
Sheep	1:3,200	1:6,400	1:3,200	1:2,400 av.	1:3,200	1:3,200
Beef	1:3,200	1:3,200	1:3,200	1:1,600	1:1,600	1:1,600
Deer	1:1,600	1:1,600	1:800	1:800	1:400	1:400
Pig	1:600 av.	1:600 av.	1:400	1:200	1:100 o	o
Horse	1:400	1:400	1:100	o	o	o
Dog	1:400	1:400	1:200	1:200	1:200	o
Monkey	1:400	1:600 av.	1:400	1:400	1:400	o

TABLE XXIII

EFFECT OF DILUTION OF ANTISERUM ON RELATIONSHIP VALUES

ANTIGEN	ANTI-GOAT SERUM: ♀ H35					
	1:1 No Dilution (Percentage)	1:2 Dilution (Percentage)	1:4 Dilution (Percentage)	1:6 Dilution (Percentage)	1:8 Dilution (Percentage)	1:10 Dilution (Percentage)
Goat	100	100	100	100	100	100
Sheep	50	100	50	37.5	50	75
Beef	50	50	50	25	25	37.5
Deer	25	25	12.5	12.5	6.25	9.4
Pig	9.4	9.4	6.25	3.12	0.78	o
Horse	6.25	6.25	1.56	o	o	o
Dog	6.25	6.25	3.12	3.12	3.12	o
Monkey	6.25	9.4	6.25	6.25	6.25	o

rather than of kind. Group reactions persist even with dilutions of 1:10. Extra-specific group reactions were eliminated entirely in ♀H35 but not in ♂C11. A probable reason is that the former was a much weaker antiserum.

A very significant fact is that dilutions of 1:2 are without effect and, second, that the readings were more clear than with the non-

diluted serum. The quantity of antiserum is an important item in relationship testing; and the quantity can be increased twofold by dilutions, thus permitting a much larger series of tests to be made. Tests with dilutions higher than 1:10 are being carried out at the present time.

4. *Effect of age on precipitins.*—It is known that antitoxins may be preserved for a long time (Wells) when dried completely, but that in fluid serum they gradually disappear. Less is known about the retention of potency of precipitins. Hektoen and Boor (1931) found that changes may take place in precipitin sera on standing. These

TABLE XXIV

EFFECT OF AGE OF ANTISERA ON TITER AND RELATIONSHIPS

ANTIGEN	♂ A ANTI-BEEF SERUM, PRODUCED NOVEMBER, 1928			
	Titer		Relationships	
	April, 1929	March, 1932	1929 (Percentage)	1932 (Percentage)
Beef	1:6,400	1:1,600	100	100
Sheep	1:3,200	1:800	50	50
Pig	1:1,600	1:200	25	12.5
Horse	1:800	1:400	12.5	25
Man	1:800	1:200	12.5	12.5
Cat	0	0	0	0

changes are both qualitative and quantitative. They reported that serum against chicken, guinea-hen, and turkey hemoglobins were active, when nearly 3 years old, against chicken, goose, and turkey hemoglobins, but lost their reactions to guinea-hen, pigeon, and mammalian hemoglobins. Gara (1929) found that the age of $2\frac{1}{2}$–5 years markedly affected seventeen precipitating sera, while three were unaffected.

The effect of age on four antisera is recorded in Table XXIV, XXV, XXVI, and XXVII. The antisera were approximately 3 years old, and the homologous and heterologous titers of the fresh and older sera are given as are the relationship values. The serum was kept in a cool place the entire time with the exception of approximately 1 week during transportation from New Jersey to Wisconsin.

TABLE XXV

EFFECT OF AGE OF ANTISERA ON TITER AND RELATIONSHIPS

ANTIGEN	♀ B ANTI-PIG, PRODUCED NOVEMBER, 1928			
	Titer		Relationships	
	March, 1929	March, 1932	1929 (Percentage)	1932 (Percentage)
Pig	1:25,600	1:12,800	100	100
Beef	1:6,400	1:400	25	3.12
Sheep	1:6,400	1:200	25	1.56
Horse	1:800	1:200	3.12	1.56
Man	1:800	1:400	3.12	3.12
Cat	1:1,600	0	6.25	0

TABLE XXVI

EFFECT OF AGE OF ANTISERA ON TITER AND RELATIONSHIPS

ANTIGEN	♀ D ANTI-BEEF, PRODUCED JANUARY, 1929			
	Titer		Relationships	
	March, 1929	March, 1932	1929 (Percentage)	1932 (Percentage)
Beef	1:6,400	1:1,600	100	100
Sheep	1:2,400*	1:400	37.5	25
Pig	1:2,400*	1:400	37.5	25
Horse	1:1,200*	1:400	18.75	25
Man	1:300*	1:200	4.67	12.5
Cat	1:150*	0	2.33	0

* Average reading.

TABLE XXVII

EFFECT OF AGE OF ANTISERA ON TITER AND RELATIONSHIPS

ANTIGEN	♀ F ANTI-HORSE SERUM, PRODUCED JANUARY, 1929				
	Titer			Relationships	
	February, 1929	March, 1932 (Original Antigen)	March, 1932	1929 (Percentage)	1932 (Percentage)
Horse	1:25,600	1:12,800	1:3,200	100	100
Beef	1:3,200	0	1:100	6.25	3.12
Sheep	1:400	0	1:100	1.56	1.56
Man	1:200	0	1:100	0.78	3.12
Cat	1:400	.....	0	1.56	0
Pig	1:400	.....	0	1.56	0

The potency of all the sera has been reduced greatly. The results are fairly constant with exceptions of the homologous tests of the anti-pig and anti-horse sera. ♀ F showed a high homologous titer only when the original antigen was used. The protein concentrations of the two test antigens were about equal, and the divergence of reaction could not be accounted for on these grounds. Is it possible that an isoprecipitin reaction was the cause of this? Only two heterologous tests were made against original antigens—in both cases complete specificity resulted. This suggests that an antigen, upon standing, may lose its power to precipitate a heterologous antiserum *in vitro*. This can be correlated with the lesser production of heterologous antibodies *in vivo*, as previously stated.

The two sera which were the stronger originally showed the greater decrease in loss of titers, especially for cross tests. An explanation which seems fitting is that the heterologous reactions in these two cases are concerned with more distantly related animals and that bloods of such forms lose their cross precipitating power very readily. The relationship values given by the two anti-beef sera were altered but slightly, while the other two showed great differences.

Although there is still need for further data, one can conclude that age results materially in loss of precipitins, regardless of the strength of the original serum. This must be considered in medico-legal work.

Age of antiserum also affects relationship values, and the use of serum older than two years is not advised for such work.

5. *Absorption tests.*—Wolfe in 1929 reported a method for distinguishing the individual bloods of a mixture. This differentiation was possible because of the special absorption of precipitin solutions. Various antisera, after being absorbed with heterologous serum, reacted only with the homologous serum when a mixture of two bloods was involved. The purpose of the records shown in Tables XXVIII–XXXII, inclusive, is to throw more light on the absorption phenomenon. Does an antiserum, treated with an antigen, lose only antibodies for the specific antigen? Would absorption of an antiserum by very closely related proteins increase the specificity of the serum?

The absorption method consisted of adding 4 cc. of antisera to 16 cc. of a 2 per cent antigen solution. When done in this manner, the loss of titer of the antiserum could hardly be due to dilution

effect, for work already illustrated shows that diluting the antiserum fourfold causes but a very slight loss of titer. The antigen-antibody mixture was incubated for 1 hour at $37.5° \pm 1°$ C. and centrifuged after 24 hours in the ice box. The supernatant fluid was used as the antiserum.

Table XXVIII contains the normal reactions of ♀H205, an anti-human serum, and also the reactions of this serum after absorption with *Macacus* blood. Not only were all the antibodies for *Macacus* removed but also those for baboon, beef, and pony. Much weaker reactions occurred with human-being and chimpanzee serum proteins.

TABLE XXVIII

ABSORPTION TESTS

ANTIGEN	ANTI-HUMAN SERUM: ♀ H205	
	Normal	Absorbed with *Macacus*
Man	1:25,600	1:1,600
Chimpanzee	1:19,200*	1:800
Macacus	1:12,800	0
Baboon	1:12,800	0
Beef	1:800	0
Pony	1:3,200	0

* Average reading.

Table XXIX records the reactions of primate bloods with normal anti-human serum and anti-human serum absorbed with chimpanzee blood. The latter antiserum failed to react with baboon or *Macacus*, and reacted only very weakly with chimpanzee. The human blood could readily be distinguished from chimpanzee. These results are of great medico-legal value and are reliable when proper controls are made.

In Table XXX are found a series of titers of a normal and an absorbed anti-sheep serum, beef being used as the absorbing antigen. This absorption produced an extra-species specificity in only two instances. Beef could now be more readily distinguished from goat or sheep serum. The beef took out most of the antibodies for deer, revealing probably a closer similarity to deer than to sheep or goat.

The antibodies for pony, monkey, and man were not all removed, although these animals are much more distantly related to the sheep

than is beef. The cross reactions of the original antiserum may not have been due to a single antibody but to more than one. One of these antibodies may have been common to all the heterologous sera involved, and was removed by absorption with the beef. The other antibodies were not common to beef, and remained as beef took out all its own antibodies, which included the one which reacted with all the sera.

TABLE XXIX

ABSORPTION TESTS

Antigen	Anti-human Serum: ♀ H205	
	Normal	Absorbed with Chimpanzee
Man	1:25,600	1:1,600
Chimpanzee	1:19,200*	1:300*
Macacus	1:12,800	0
Baboon	1:12,800	0

* Average reading.

TABLE XXX

ABSORPTION TESTS

Antigen	Anti-sheep Serum: ♂ P21	
	Normal	Absorbed with Beef
Sheep	1:12,800	1:3,200
Goat	1:9,600*	1:3,200
Beef	1:6,400	1:600
Deer	1:6,400	1:200
Pig	1:800	0
Pony	1:1,600	1:400
Cat	1:400	0
Monkey	1:800	1:300*
Man	1:400	1:200

* Average reading.

Table XXXI represents reactions of a normal anti-goat serum, and this serum after absorption with beef antigen. An extra-group specificity was produced. It appears rather inconsistent to find the reaction with sheep diminishing so greatly. Although the antiserum gave a slight group reaction after absorption, it could easily differentiate beef, deer, or sheep serum proteins from goat. The original

antiserum was rather weak, and this may be the reason for the greater species-specificity produced.

The next table (XXXII) records tests for fox antiserum ♀H33 which was also absorbed with beef No. 11. This absorbed antiserum did not react with beef, and all other ungulate reactions were lower.

TABLE XXXI

ABSORPTION TESTS

ANTIGEN	ANTI-GOAT SERUM: ♀ H35	
	Normal	Absorbed with Beef
Goat	1:6,400	1:1,600
Sheep	1:3,200	1:400
Beef	1:3,200	1:200
Deer	1:1,600	1:200
Pig	1:600	0
Horse	1:400	0
Dog	1:400	0
Man	1:200	0

TABLE XXXII

ABSORPTION TESTS

ANTIGEN	ANTI-FOX SERUM	
	Normal	Absorbed with Beef
Dog	1:9,200*	1:9,200*
Cat	1:1,600	1:1,600
Badger	1:1,600	1:1,600
Beef	1:1,200	0
Goat	1:800	1:400
Pig	1:1,600	1:400
Sheep	1:1,600	1:400
Deer	1:100	0
Man	1:400	1:400
Chimpanzee	1:800	1:400

* Average reading.

The titers of the carnivores and primates were not reduced, again suggesting the presence of multiple antibodies.

6. *Medico-legal applications of results obtained.*—Wolfe (1929) showed that, for the "trial-and-error" method of identification of unknown bloods, a shorter and less cumbersome technique could be substituted. This method was based on relationship values previ-

ously obtained. It has been suggested by Dr. Boyden, of Rutgers University, that a "key" could be prepared for various blood protein reactions which would elucidate further this short procedure. A key could be made for each different species of antisera.

The results secured with specific absorption tests can be of great aid in differentiating closely related animals. From these results and the relationship values of ♀H205 and ♀H35, two keys have been made, which are shown in Tables XXXIII and XXXIV, respectively. By following these tables and using proper technique and controls, the results should be dependable.

TABLE XXXIII

KEY TO ANTI-HUMAN SERUM REACTIONS

A. Reaction 50–100 per cent ..... Higher primates
  B. (Antiserum absorbed with monkey)
    Reaction 50–100 per cent ..... Man, chimpanzee
    C. (Antiserum absorbed with chimpanzee)
      Reaction 100 per cent ..... Man
    CC. Reaction less than 50 per cent ..... Chimpanzee
  BB. No reaction ..... Monkey, baboon
AA. Reaction less than 50 per cent ..... All other sera

TABLE XXXIV

KEY TO ANTI-GOAT SERUM REACTIONS

A. Reaction 50–100 per cent ..... Goat, beef, sheep
  B. (Anti-goat absorbed with beef)
    Reaction 50–100 per cent ..... Goat
  BB. Reaction less than 50 per cent ..... Beef, sheep
AA. Reaction less than 50 per cent
  B. Reaction 25 per cent ..... Deer
  BB. Reaction less than 9 per cent ..... Horse, man, pig, etc.
    (To determine further must refer to additional tables)

The "keys" presented are brief and incomplete. Keys could be constructed for all the antisera with the tests made and with several more absorbed antisera. The evidence submitted shows that they can be of value and, when used properly, may yield reliable results. In criminal trials that involve unknown blood stains, two points may arise: first, does the court desire to know the ultimate source of the stain, or does it wish to know if the stain was caused by the blood of one particular animal? The latter case would involve but one "key," while the former would involve more, and hence the need of several tables by the medico-legal expert.

DISCUSSION

The relationship values of twelve species of mammals (reacted against twenty-three species) obtained in the preceding experiments agree quite closely with those of Boyden and Nuttall and also within themselves. The reciprocal relationship tests, devised by Boyden, verified the results in a large majority of the cases, but many of the reactions involving serum or antiserum of carnivores were anomalous. It was suggested that the possible cause of this irregularity was due to the diet of the carnivorous animals.

The cat's and dog's food had consisted chiefly of beef and sheep meat, while the fox's also included horse meat. It is possible that these ungulate proteins, or that part of the protein molecule which may cause the production of a very small amount of homologous antibodies, is found in the circulating blood after the animal's meal. This blood, when injected into a rabbit, could incite both homologous and heterologous antibody formation. If this deduction is true, it would be important to starve the carnivorous animals for a short time before bleeding, to allow for a disappearance of the absorbed food from the blood stream. The deviations could then be tested, for the dog, fox, or cat's blood should now only produce immune bodies caused by their respective serum proteins. On the other hand, is it not probable that the irregularities are due to an inhibition of the reaction when anti-ungulate and carnivore serum are brought together? This condition might possibly be tested by analogy. By adding very slight amounts of dog or fox serum to beef serum and reacting this mixture with the anti-beef serum, one could determine whether or not an inhibition occurs. However, if this inhibition should not occur, the foregoing assumption is not necessarily incorrect.

A great deal of verification of results is important in this type of research. Accordingly, a comparison of antisera reactions of very closely related forms was offered as another procedure in verification of results. The comparison is based on the assumption that the chemical constituents of the related animals are very similar and that hence the reactions they produce should be alike. The results of comparison justify this type of corroboration.

Besides the confirmation of the work of other authors, additional serological classification data is given. The author knows of no previ-

ous quantitative relationship studies of fox, cat, *Macacus*, baboon, deer, or pony bloods. The results obtained with the antisera produced by these bloods confirm existing classification based on comparative anatomy and embryology.

It should be noted in most of the tests that an antiserum shows a similar degree of relationship to several animals. Take, for example, the anti-beef (♀ UV12) reactions which show ten cross reactions between 3.12 per cent and 6.25 per cent, inclusive; three below 3.12 per cent; and the rest 12.5 per cent or above. Does this mean that the ten animals are all equally related to beef? It can mean that; but it does not follow that these animals are very closely related to each other, for it would be preposterous to assume close affinity of human being, fox, horse, and pig. These latter four animals may be equally related to the cow, for all five animals have had a common phylogeny at first and then they probably all diverged in different directions in the development of their own race. The results emphasize that a phylogenetic tree of mammals must be made, if at all, on a three-plane dimension basis (Boyden, 1932).

The quantity of antiserum plays an important part in serological classification, for it governs the number of reactions that can be made. Rabbits, which are most often used for antibody production, yield approximately 60–100 cc. of serum. When duplicate and triplicate tests are made and controls used, only a limited number of such tests can be performed. It has been found that a 1:2 dilution of antiserum with buffered salt solution does not affect relationship values. By diluting the antiserum, one could increase greatly the number of reactions and extend the usefulness of the results. Then, again, dilutions of antiserum often make the readings of the "ring" more definite. At times certain antisera react peculiarly, in that at the point of contact of serum and antiserum a "non-specific" ring is formed in all tubes, including the control. This condition disappears with a dilution as low as 1:2.

Antisera produced by the same species of blood may not yield similar relationship data. This discrepancy is believed to be due to the inherent variability differences in response of the antibody producers. Careful selection of animals may be of aid. In serological classification it would be wise to use only those reciprocal values which are within the range of error.

The complete species-specificity of an antiserum would be the ideal condition for medico-legal purposes. Antisera against different tissues or parts of tissues of the body have not yielded this desired result. Yet the precipitin test, when placed in the hands of a reliable expert, can render evidence which is trustworthy and which courts could accept.

It is known that a high titered and clear antiserum is desirable and necessary. The determination of protein concentration of unknown blood is advisable; and proper controls are always essential, especially when mixtures of bloods are to be identified. The results of this paper bear additional information on the medico-legal aspect of the precipitin reaction.

Antiserum, when used, should not be too old. Serum up to 2 years of age seems to have deteriorated but slightly and is reliable; but after standing 3 years, serum shows marked deviations from original reactions. The potency of the antiserum decreases considerably; and thus, where once a 1:1600 titer meant a reaction with a distantly related animal protein, it may now result only from a homologous antigen. The antigen itself, in so far as homologous reactions are involved, loses none of its properties.

An antiserum diluted from 1:5 to 1:10 shows a greater species-specificity than the normal non-diluted antiserum, but not enough to be of much greater use than normal antiserum in forensic medicine. The reactions of the more closely related forms is barely affected by dilutions of 1:10. If still higher dilutions will produce a greater species-specificity, it could be of medico-legal value. In producing specificity, dilution affects the low titer serum more than the strong serum.

It has been shown that specific absorption of an antisera by a heterologous antigen removes the antibodies for the heterologous substance. Further work on the absorption phenomenon has demonstrated that it produces a great species-specificity, for, not only are cross reactions with the particular antigen eliminated, but reactions with equally related animal bloods are also lost. Nishegorodzeff (1930) demonstrated almost similar results by injecting heterologous antigens just before bleeding into the rabbit producing antibodies.

Landsteiner and Van der Scheer (1924) and Landsteiner and

Miller (1925), using absorbed agglutinins against erythrocytes, found they could distinguish between human and chimpanzee bloods. Of course, this could not be of medico-legal application unless very fresh blood specimens were on hand. The tests enumerated in this paper seem to indicate that this distinction can be made with the precipitin test. Human antiserum, after absorption with chimpanzee blood, reacted very faintly with the latter but much more strongly with human antigen, and did not react with *Macacus* or baboon bloods after being absorbed with *Macacus* serum proteins. Beef could easily be distinguished from goat or sheep bloods, to which it is very closely related, by this method.

It may be emphasized that by the absorption procedure one can determine the degree of relationship of individual animals within a closely related group. Sheep was shown to be more closely related to goat than beef or deer, and chimpanzee more closely related to man than *Macacus* or baboon.

The author wishes to state that, although accurate procedures were followed in all this work, yet the amount of data on dilution effects and specific absorption is not sufficient. Tests are being carried out which he feels will further verify results and may add other important conclusions.

SUMMARY

1. Relationship values of other authors have been confirmed.

2. Additional relationship values were obtained which substantiate relationships secured by comparative anatomy.

3. Reciprocal relationships have verified the results of the majority of tests, with the exception of tests involving carnivores.

4. A possible cause of this anomaly of Carnivora serum has been suggested.

5. Another method of verification of results was offered.

6. A 1:2 dilution of antisera does not alter relationship values.

7. A 1:2 dilution of antiserum often makes readings more precise and increases the number of tests that can be made.

8. Higher dilution affects relationship values, but even a 1:10 dilution does not produce extra-group specificity or at times extra-species specificity.

9. Age of 3 years decreases the potency of an antiserum, and such antiserum should not be used for medico-legal purposes.

10. Specific absorption of antiserum results in an antiserum which is most reliable in distinguishing even very closely related animals.

11. A "key" has been made involving relationship values and specifically absorbed antisera which defines a shorter procedure for identification of unknown bloods.

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QUANTITATIVE STUDIES ON PRECIPITINS

(Sixteen figures)

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ALTHOUGH the precipitin reaction has been employed by a great number of investigators in the study of animal relationships and related problems, only a few have thought of its use in quantitative terms with a known range of error. Since the quantitative volumetric precipitin technique of Boyden and Baier (1929) has proved to be fairly simple, rapid, and trustworthy, it was thought advisable first to further investigate some properties of this biological reaction, so as to form a more secure basis for future relationship studies.

HISTORICAL REVIEW

The discovery of the precipitin reaction by Rudolph Kraus (1897) opened a new era of immunological study. He found that the blood serum of a goat previously injected with sterile cholera culture filtrates had the power of producing a precipitate when mixed with clear solutions of cholera filtrates. The method of determining the degree of reaction, as practiced by Kraus, consisted in the formation of flocculent particles by the mixture of various amounts of antigen and antiserum in small glass test tubes. The titer, or strength, of an antiserum was determined by the highest dilution of antigen in which a definite amount of antiserum would just show a definite reaction when incubated at a known temperature (usually 37° C.).

The scope of the reaction was later broadened by Bordet (1899) and Tchistovitch (1899), when it was found that proteins other than bacterial products could be used. Bordet injected fowl blood into rabbits and rabbit blood into fowls and obtained specific precipitating antisera in each case. Tchistovitch, using eel serum and horse serum, likewise obtained specific antisera in each case. These investigators still employed the flocculation reaction of Kraus.

Following on these early studies, many investigators found that the reaction could be used with proteins in general, regardless of

their origin. Also, it was demonstrated (first by Uhlenhuth in 1901) that, while the reaction is not entirely specific, proteins will react with an antiserum in a degree corresponding generally to their relationships. Hence we have the basis for the application of precipitins to the study of the blood relationships of animals (Nuttall, 1904, and Boyden, 1926).

Ascoli (1902) modified the original flocculation technique of Kraus by placing the heavier antiserum beneath the antigen dilution somewhat after the fashion of Heller's nitric acid test. The layer, or "ring," of precipitate that formed at the junction was observed; and, as in the previous flocculation test, the tube, containing the highest dilution of antigen, yet still showing a ring formation at the end of a definite time, was taken as the titer.

Nuttall (1904) modified the flocculation test so as to make it quantitative. He suggested measuring the amount of precipitate formed in capillary tubes. At first sealed capillary tubes were used, with which many difficulties arose. Among the sources of error were: (1) differences in the bore of the tubes which affected the degree of packing of the precipitate, and (2) errors of dilution of varying protein concentrations. This technique was used to some extent by Graham-Smith and Sanger (1903), who determined the range of experimental error to be 10 per cent. The results of Nuttall and Strangeways (1904) (see Nuttall, 1904), on the other hand, suggest a much greater variation than this. Nuttall and Inchley (1904) have modified this method, and state that they have greatly improved the technique so that more uniform results can be obtained. Calibrated capillary tubes of equal bore have been used. Their technique consisted in the mixture of 0.5 cc. of a 1:21 dilution of serum and 0.1 cc. of antiserum. This was allowed to stand 24 hours, after which the mixture was drawn up in the capillary tubes, sealed off with mercury, and allowed to stand for 2 days. At the end of this period the volume of precipitate was measured in a special calibrated apparatus.

In 1904 Schur used the centrifuge to aid in the packing of the precipitate to be measured. He made paired readings, centrifuging at a known rate of speed for 20 minutes. This was further modified by Hamburger (1905) to consist of centrifugalization to constant volume at 2,500 r.p.m. The tubes were sealed at the bottom, however,

and the entire reaction took hours to perform. The use of this method by Hamburger and Arrhenius (1906) in the study of the effect of varying proportions of antigen and antibody on precipitate production is a step in advance. Here they used a constant temperature of 37° C. Tests made in pairs show almost perfect agreement when constant volume is reached. Mollison (1924) has also measured the precipitate volumetrically in which he centrifuged to a "certain minimum" for a known period.

Of all these experimenters, Graham-Smith and Sanger, and Nuttall were the only ones to attempt a statement of the amount of error in their technique. Boyden and Baier (1929) have recently shown that the volumetric measurement of amount of precipitate can be made quite rapidly and with a fair degree of reliability. They are the first to use the probable error as a measure of reliability in precipitin tests. Only standard calibrated instruments were used, and the conditions of reaction were kept constant for all similar measurements.

With regard to the work done on the formation of precipitates as influenced by temperature, pH, salinity, etc., very little is to be found in the literature. The effect of temperature on the titer of an antiserum and on the antigen previous to union is reported by Graham-Smith (1903), who used a volumetric technique. He found that a constant volume of precipitate could be obtained from an undiluted antiserum having been previously heated to as high as 50° C. From 55° to 62° C. a marked diminution in volume occurred. Myers (1900) stated that the reaction took place rapidly at 37° C; and this has been confirmed by Wassermann and Schütze (1901) and Michaelis (1902), while Stochis (1902) considers 40°–42° C. as most favorable. Biondi (1902) found the temperature to exert a considerable influence, while Linossier and Lemoine (1902) obtained reactions from 0° to 58° C. Still others (Kister and Wolff, 1902) state that there is no marked difference in the reaction at room temperature and 37° C. Strangeways (1903) (see Nuttall, 1904), using the quantitative technique of Nuttall, has shown that the formation of precipitate is more rapid at room temperature than at 0° C., and still faster at 37°C., but that finally the quantity of precipitate formed in each case is the same.

As to the effect of salt concentration, there is little agreement. Linossier and Lemoine (1902) found that even 1 per cent of salt impeded precipitation and that 5 per cent prevented it. Eisenberg and Volk (1902) found that even as much as 18 per cent of salt has no influence, and Rostoski (1902) came to a similar conclusion when using 10 per cent salinity. According to Graham-Smith and Sanger (1903), a slight decrease in volume of precipitate occurred up to 7 per cent salinity, and on further increase in salt an increase in volume of precipitate followed. However, they also noticed that at these high salt concentrations the precipitate was more flocculent and, owing to the increased specific gravity of the medium, took longer to settle.

As to the effect of hydrogen ion concentration, it is known that precipitation will occur within a wide range of the neutral point. Graham-Smith and Sanger (1903) showed volumetrically that even small quantities of acid or alkali reduce the quantity of precipitate formed without altering the specificity of the reaction. Hirsch (1923) found that the hydrogen ion range of sheep serum and homologous immune serum lies between pH of 6.0 and 9.4. He also determined that maximum precipitation occurs near the acid side of the range—near the iso-electric point of the protein fraction precipitating from sheep serum. This seems to agree approximately with the pH range of agglutinins as determined by De Kruif and Northrop (1923), who used a suspension of *Bacillus typhosus* with its immune serum, which was found to be from pH 3.7 to 9. Mason (1922) found that a specific precipitate will occur between pH of 4.5 and 9.5; also, that a specific precipitate allowed to form in a neutral medium will be dissolved if the pH is reduced to less than 4.5 or increased to more than 9.5. However, Mason determined pH colorimetrically and used the flocculation test to determine the formation of precipitate. Evans (1922) showed that the hydrogen ion concentration has an effect on serological studies and recommended a buffered physiological salt solution as diluent where comparable results are desired.

PURPOSE OF THIS INVESTIGATION

The purposes of this investigation are: (1) to study quantitatively the volumes of precipitate formed in the precipitin reaction; and (2)

to determine the effects of factors that may influence these volumes in formation and measurement.

MATERIALS AND METHODS USED IN THIS INVESTIGATION

A. PREPARATION OF ANTIGENS

Simple and complex animal proteins have been used as antigens. The simple proteins used are: (1) crystalline egg albumin, and (2) oxyhemoglobin. The complex protein antigens are (3) blood serum.

The crystalline egg albumin has been prepared according to the method of Hopkins and Pinkus (1898). Briefly, this consisted in the repeated crystallization of the albumin fraction with a saturated solution of ammonium sulphate. Crystallization was repeated three times. The aqueous solution of the purified crystals was then dialyzed, using parchment paper or collodion membranes, to remove the inorganic salts. If the final protein solutions were to dilute, they were concentrated by evaporation under an electric fan.

The oxyhemoglobin was prepared after the method of Marshall and Welker (1913). This consisted in washing the erythrocytes several times with physiological saline, laking the cells with ether, and removing the stroma and other proteins with aluminum cream. The oxyhemoglobin was then dialyzed to remove any inorganic salts. After filtration for sterility (Seitz filters), the solutions were stored in 5 cc. ampules in the ice box until ready for use. The serum was collected after clotting of the blood, and then likewise filtered and stored in ampules.

All dilutions of proteins, using physiological buffered salt solution as diluent, have been brought to a pH of 7, determined colorimetrically (according to the method of Evans, 1922).

The concentration of the proteins used in this investigation has been determined by making modified Kjeldahls on 10 cc. samples of a dilution of the antigen according to the method of Folin and Wright (1919).

B. PRODUCTION OF ANTISERA

Increasing dosages of from 1 cc. to 5 cc. were injected intravenously into rabbits at 3-day intervals for 15 days for precipitin production. In the case of the oxyhemoglobins, which often proved toxic to the animals, the last three injections of 3 cc. to 5 cc. were given

intraperitoneally. Eight days after the last injection a preliminary test was run on a 5-cc. sample obtained from the median ear artery to determine the titer of the antiserum. If the antiserum did not react with the antigen in a dilution of at least 1:6400, the injections were continued. Having obtained the desired potency of serum, the rabbits were anesthetized and bled completely, from the heart, on the tenth day, by means of a syringe. The blood was allowed to clot overnight, centrifuged, and the serum obtained, filtered through Seitz filters, and stored in 5 cc. ampules in the ice chest. Fifteen vials are usually obtained from a normal rabbit.

C. TECHNIQUE OF PERFORMING REACTIONS

The technique used in this investigation consisted in preliminary titer determinations to ascertain the strength of the antiserum. The ring test was used for this analysis, from which also the zone of optimal proportions, to be mentioned later, can be seen. From this it is possible to judge the range of antigen and antibody proportions to produce a suitable amount of precipitate for quantitative measurement.

The quantitative technique as described by Boyden and Baier (1929) has been used throughout this investigation, with modifications to determine the effects of various factors on the precipitin reaction. The technique is as follows: "(*a*) measure into clean glass tubes (75×10 mm. serological tubes with bottoms heated and flattened to just fit the lower end of the thrombocytocrit) definite amounts of antigen and antiserum; (*b*) mix immediately and place in the water bath at 37.5° C. for 1 hour; (*c*) draw up the precipitate suspended in the supernatant fluid into Van Allen thrombocytocrits, seal with spring clips, and centrifuge at 2,400 r.p.m. for two 15-minute periods, reading the absolute volume at the end of each period; (*d*) remove clips, blow out the contents of the thrombocytocrits, and wash by sucking up in succession, saline solution, distilled water, alcohol, and ether. Dry over a lamp or radiator."

EXPERIMENTAL RESULTS

Experiment I. The volume of precipitate obtained, as influenced by quantity of antigen in the reaction.—This experiment was designed to

test the relationship of the volume of precipitate obtained to the quantity of antigen present, using a constant amount of antiserum (0.5 cc. of the dilution indicated) for each study. The antigen was varied in dilution, as shown on the abscissas of Figures 1–8, but the volume remained constant at 0.5 cc. The total incubation mixture was thus 1.0 cc. To insure the reliability of the results, quartet readings were obtained at each dilution of antigen and the results plotted

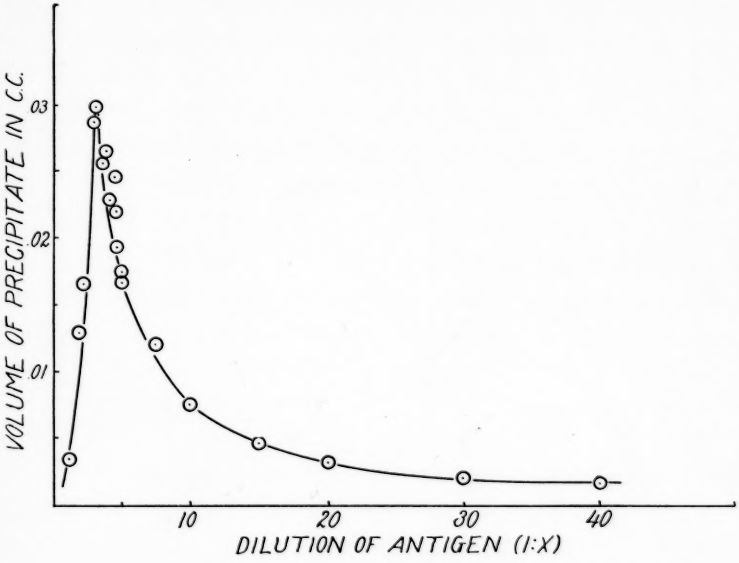

FIG. 1.—Effect of antigen dilution

on a graph, with the ordinate representing the actual volume in cubic centimeters of precipitate obtained.

Four graphs representing crystalline egg albumin as antigen are presented. Each graph represents a different antiserum. Figure 1 shows the relationship between antigen and antiserum, when using 0.5 cc. of crystalline egg albumin (sample A), diluted as shown, as antigen, and 0.5 cc. of antiserum (rabbit ♂-21) diluted 1:5. In Figure 2 the antiserum (rabbit R-19) was constant, when using 0.5 cc. of a 1:2 dilution reacting with crystalline egg albumin antigen (sam-

ple 22), and using 0.5 cc. of a dilution as shown. Likewise, Figure 3 represents the reaction between 0.5 cc. of antiserum (rabbit 21-W) diluted 1:2 against 0.5 cc. of crystalline egg albumin antigen (sample 21) diluted as indicated on the graph.

Two graphs representing the relationship of antigen to antibody, when a simple protein, oxyhemoglobin, is used, are presented. Figure 4 represents the reaction obtained using 0.5 cc. of antiserum

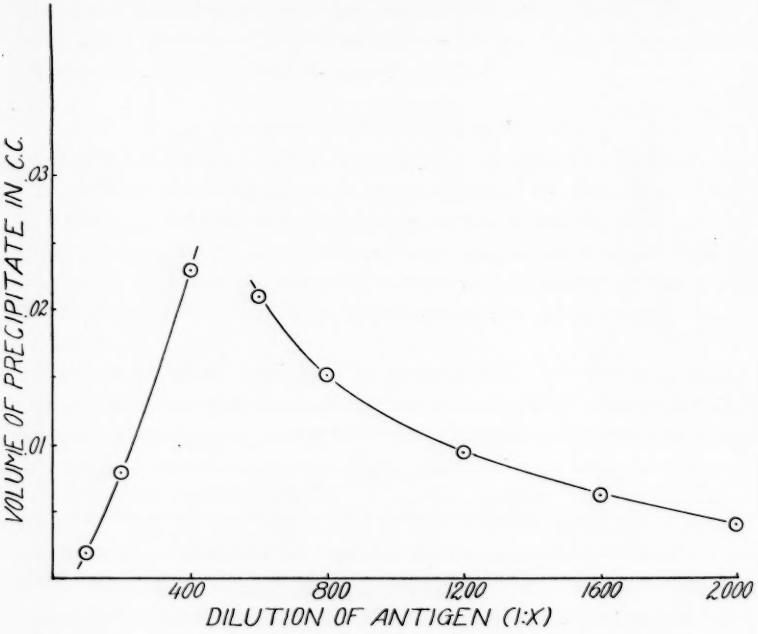

FIG. 2.—Effect of antigen dilution

(rabbit Y-70) undiluted, against 0.5 cc. of varying dilutions of beef oxyhemoglobin antigen (sample 40) diluted as indicated. In Figure 5 is shown the result of reacting 0.5 cc. of antiserum (rabbit 26-W) diluted 1:2 against varying dilutions of 0.5 cc. of dog oxyhemoglobin (sample 1) as antigen.

Three graphs representing complex protein antigens reacting with their homologous antisera are presented. Figure 6 shows the rela-

tionship between 0.5 cc. antigen (beef serum, sample 200) diluted as shown and 0.5 cc. antiserum (rabbit 9-W) diluted (1) for upper graph, 1:2, and (2) for lower graph, 1:4. In Figure 7 is shown the reaction between 0.5 cc. antigen (dog serum, sample 1) diluted as shown on the abscissa, against 0.5 cc. antiserum (rabbit 7-W) diluted at 1:5. Figure 8 shows the relationship between antigen (sheep serum, sam-

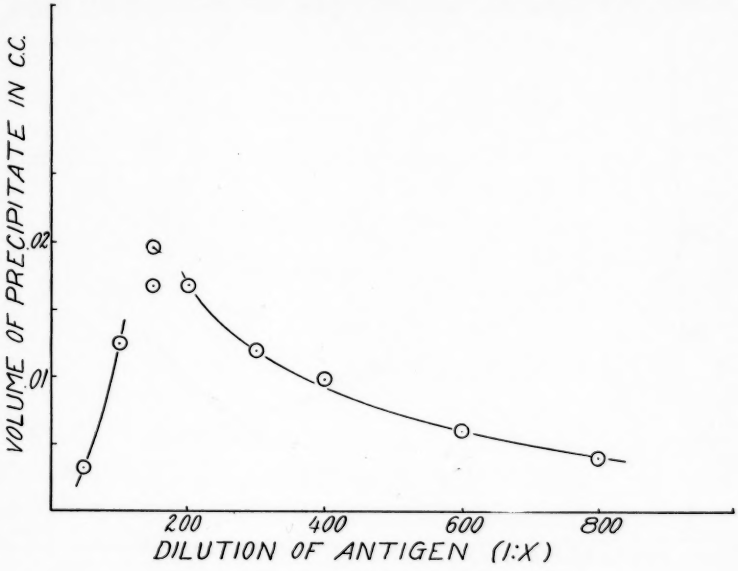

FIG. 3.—Effect of antigen dilution

ple 1), when using 0.5 cc. of the dilution shown, and antiserum (rabbit 28-W), when using 0.5 cc. of a 1:4 dilution.

From the general trend of these eight graphs, where simple and complex protein antigens are used, it can be seen that there is a decided relationship between the amount of antigen present and the volume of precipitate obtained. As the antigen concentration increases, there is a rise in the volume of precipitate obtained until a peak is reached. This is generally known as the point of maximum precipitation and occurs near the zone of "optimal proportions" of antigen and antibody. As the amount of antigen increases beyond

this point, there is a sudden decrease in the volume of precipitate obtained. This is the zone of inhibition, which illustrates the "zone phenomenon."

It will be noticed from the graphs that the abscissas show *dilutions* of antigen and not actual protein values. This was adopted so as to be in accord with the usual technique of studying the precipitin reaction. It is apparent, then, that a great deal of error might be en-

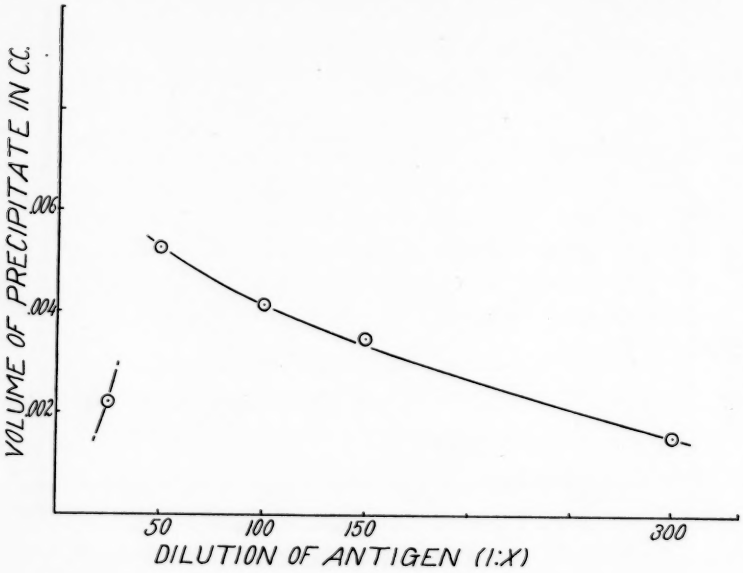

FIG. 4.—Effect of antigen dilution

countered in trying to compare precipitin reactions by such a means as studying the zone of maximum precipitation. Several difficulties will arise here: (1) that of inability to find the exact dilution at which it occurs; (2) the fact that the change in protein content becomes progressively greater as we progress from higher to lower dilutions of antigen; and (3), from a volumetric standpoint it is difficult to measure, with any degree of reliability, the exact volume of precipitate at this point.

From these graphs, which may be designated as the normal anti-

gen curves, it is possible to choose various dilutions of antigen to produce the volume of precipitate desired. Dilutions may be taken that will fall within the zone of inhibition, to study the effect of relative antigen excess; or they may be chosen to produce the point of maximum precipitation; or points may be chosen elsewhere to compare various antigens. If this curve is first obtained with only a few points, for an antigen, one may tell the possible range of reaction of the antigen.

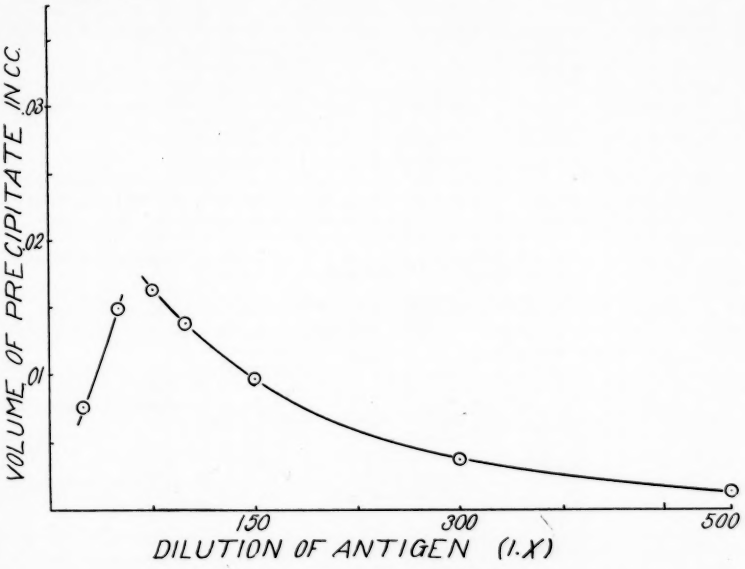

FIG. 5.—Effect of antigen dilution

Table I is given so that the degree of reliability which may be placed on the results obtained can be seen. In the table taken from the readings of Figure 3 the percentage of deviation of the most variable reading is given from the mean value. The probable errors of the mean values are also given. From these values it can be seen that some significance may be attached to the results. The probable error of any value serves as a measure of the variability of the observations from which it is derived. For example, from Table I, choosing at random a dilution of antigen of 1:800, it can be seen that

the mean equals 0.00417 ± 0.000055, which means, simply, that the chances are equal that the average of any other similar set of observations taken under the same conditions will fall within the

TABLE I

STATISTICAL DATA ON READINGS TAKEN FROM FIGURE 3

Dilution of Antigen	30-Minute Readings	Mean Value	Percentage of Deviation from Mean	Actual Deviations	Standard Deviations	Probable Error of Mean
1:50	0.00344 0.00290 0.00352	0.00326	+ 7.8 −11.0	0.00018 0.00036 0.00026	0.00027	0.000105
1:100	0.01274 0.01228 0.01246 0.01219	0.01241	+ 2.7 − 1.8	0.00033 0.00013 0.00005 0.00022	0.000297	0.000708
1:150*	0.01674 0.01647	0.01660	+ 0.84 − 0.84	0.00014 0.00013	0.000135	0.000065
1:150*	0.01956 0.01938	0.01947	+ 0.3 − 0.3	0.00009 0.00009	0.00009	0.000043
1:200	0.01701 0.01656 0.01655 0.01655	0.01671	+ 4.7 − 0.85	0.00030 0.00015 0.00006 0.00006	0.000173	0.000058
1:300	0.01102 0.01159 0.01254 0.01268	0.01195	+ 6.1 − 7.7	0.00093 0.00064 0.00059 0.00073	0.00073	0.000246
1:400	0.00973 0.00973	0.00973	0.0			
1:600	0.00655 0.00615 0.00600 0.00582	0.00613	+ 6.8 − 5.0	0.00042 0.00002 0.00013 0.00031	0.000269	0.000065
1:800	0.00395 0.00403 0.00436 0.00409	0.00417	+ 4.5 − 5.2	0.00022 0.00014 0.00019 0.00008	0.000166	0.000055

* Difficult to measure precipitate at zone of maximum precipitation.

limits set by 0.00417 ± 0.000055. The average of any set of observations is said to be of significance if it is greater than its probable error by at least 4 times.

It might be of value here to present the data concerning the antigens used—their protein concentration, along with the injection data to be used for reference.

TABLE II

PROTEIN VALUES OF ANTIGENS USED IN THIS INVESTIGATION

Sample	Serum or Protein	Date Obtained	Percentage of Protein	Date Determined
CE-20	Crystalline egg albumin	11-24-28	2.010	1- ?-29
CE-21	Crystalline egg albumin	3-10-30	3.44	1- 6-32
CE-22	Crystalline egg albumin	3-11-30	5.73	1- 6-32
Pg-50	Pig oxyhemoglobin	12- 4-28	1.806	1- ?-29
PH-350	Pig oxyhemoglobin	4-19-30	16.14	1- 6-32
Bf-40	Beef oxyhemoglobin	12-10-28	0.901	1- ?-29
BH-100	Beef oxyhemoglobin	3- 4-30	4.80	1- 6-32
Dg-Hb	Dog oxyhemoglobin	5-20-31	4.19	1- 6-32
Ll-Hb	Llama oxyhemoglobin	5-25-31	3.72	1- 6-32
Dog-1	Dog serum	4-21-30	4.287	6- 9-31
Bf-200	Beef serum	2-24-30	7.276	1- 6-32
S-1	Sheep serum	1- 8-31	6.28	1-27-31

TABLE III

COMPARISON OF TITER OBTAINED TO PROTEIN INJECTED

Rabbit	Antigen Injected	Number and Means of Injections	Total Protein Injected (Grams)	Titer of Antiserum
♂V-81	3 cc. CE-20	2 intravenously	0.060	1:12,800
♀W-80	14 cc. CE-20	6 intravenously	0.281	1:6,400
♀X-60	26 cc. Pg-50	8 intravenously	0.468	1:6,400
♀Y-70	27 cc. Bf-40	9 intravenously	0.243	1:6,400
♂4-W	6 cc. CE-21	3 intravenously	0.204	1:25,600
♂6-W	21 cc. BH-100	7 intravenously	1.008	1:1,600
♂7-W	21 cc. Dg-1	7 intravenously	0.903	1:3,200
♂9-W	21 cc. Bf-200	7 intravenously	1.533	1:12,800
♂14-W	11 cc. CE-22	4 intravenously	0.627	1:102,400
♂21-W	11 cc. CE-21	5 intravenously	0.374	1:51,200
♂25-W	15 cc. PH-350	2 intravenously 3 intraperitoneally	2.424	1:25,600
♂26-W	10 cc. Dg-Hb	1 intravenously 3 intraperitoneally	0.419	1:25,600
♂27-W	15 cc. Ll-Hb	2 intravenously 3 intraperitoneally	0.555	1:6,400
♂28-W	15 cc. S-1	2 intravenously 3 intraperitoneally	0.93	1:12,800

A. The protein values of antigens used in this investigation.—Table II shows the protein values obtained for the various antigens used in this investigation, with the addition of several others as a means

of comparison. It can be seen that great variations occur in both the purified prepared proteins and the serum proteins. Hence, in studying protein relationships, one must take into account the concentration of the protein being used.

B. Injection data, in relation to amount of antigen injected, route of immunization, and titer obtained.—Table III shows the results ob-

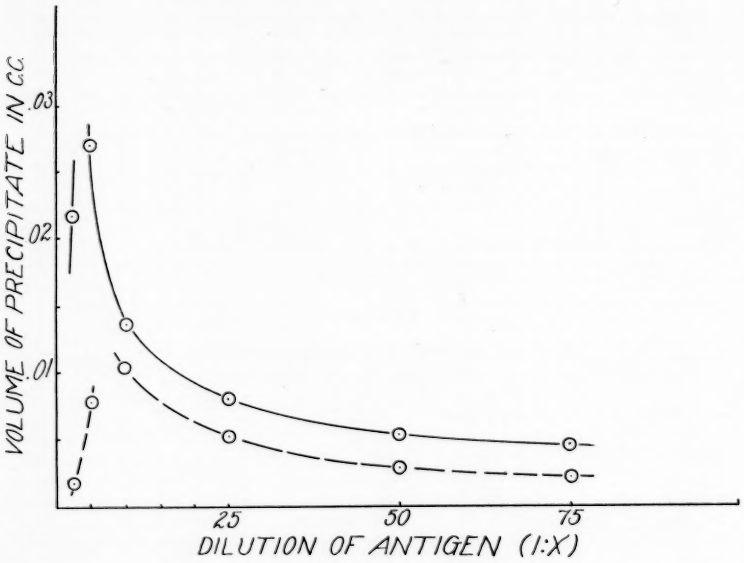

FIG. 6.—Effect of antiserum and antigen dilution

tained. It is obvious here that there is no direct relationship between the amount of protein injected and the titer obtained. Individual variations among rabbits and differences in protein antigens probably are the main causes of these variations. Several rabbits died from loss of weight, and others apparently from anaphylaxis (even though the injections were within 4 days of each other) when the oxyhemoglobin solutions were being injected intravenously. These same solutions were later given to similar rabbits except that the last three injections were given intraperitoneally. By this combined method no rabbits were lost and the titer appeared to be about as

high. However, a few cases (rabbits V-81 and 4-W) show a higher titer with only 3 cc. and 6 cc. having been given intravenously. On such a small number of rabbits as presented here, any conclusions would be insignificant.

Experiment II. Effect of dilution of antiserum on volume of precipitate obtained.—In Figure 9 is shown the relationship of antigen to

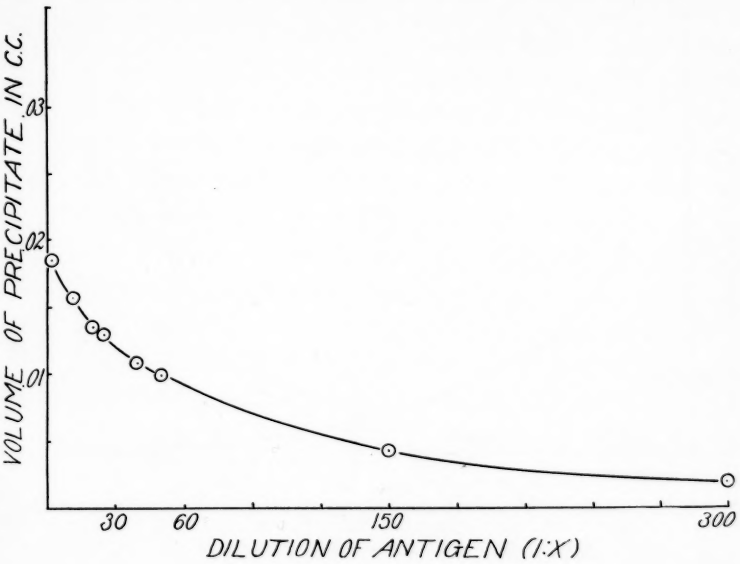

FIG. 7.—Effect of antigen dilution

antibody, using a simple protein as antigen (crystalline egg albumin, sample 22) with its homologous antiserum. The abscissa represents dilutions of antiserum (rabbit R-19), using 0.5 cc. of each dilution. The ordinate shows the volume of precipitate obtained. Four curves are given, representing four different antigen dilutions, so that the entire study shows the effect of antigen and antibody dilution on precipitate formation. Curve 1 (solid line) shows the antigen diluted 1:1200; Curve 2 (broken and dotted), antigen 1:800; Curve 3 (broken line), antigen 1:600; Curve 4 (dotted line), antigen 1:400.

From the graph it can be seen that the volume of precipitate ob-

tained is dependent on the quantity of antiserum present. This is true of all four curves. However, since Curve 1 contains less antigen than Curve 4 in the series, it can be understood from referring to Experiment 1 why, at the higher dilutions of antiserum, the curves change position on the graph. That is, the presence of the greater amount of protein antigen begins to show the solvent action of this

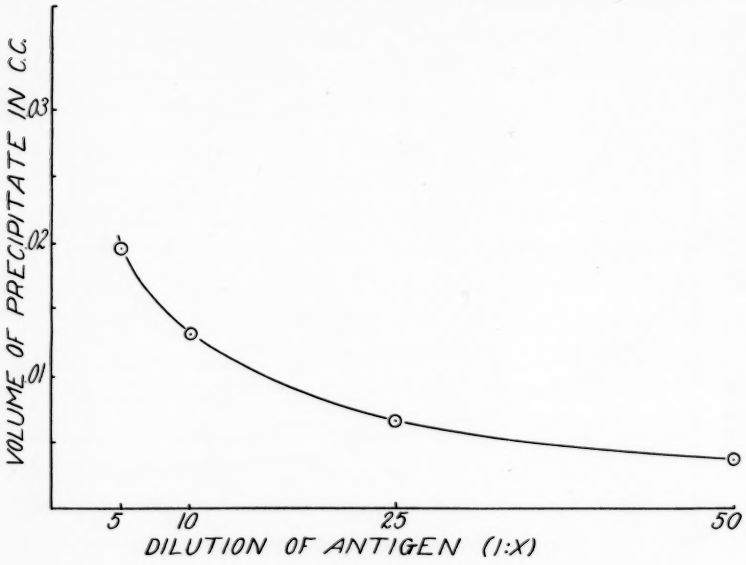

FIG. 8.—Effect of antigen dilution

excess antigen. This shows, then, that the volume of precipitate obtained depends not only on the amount of antigen and antibody present but also on the relation of antigen to antibody in the reaction. If either antigen or antibody is kept constant in amount, then the volume of precipitate is dependent on the variable constituent.

In referring to Figure 6, again, it can be seen that the same relationship exists using a complex antigen, i.e., serum.

Experiment III. Effect of temperature on formation of precipitate.—Both antigen and antibody were constant in amount, using 0.5 cc. of antigen (crystalline egg albumin, sample 20) diluted 1:150 and

0.5 cc. of antiserum (rabbit W-80) diluted 1:5; and each mixture of 0.5 cc. antigen and 0.5 cc. antiserum was incubated for 1 hour previous to centrifugation at the temperature indicated. The range of temperature was from 0° C. to 73° C.

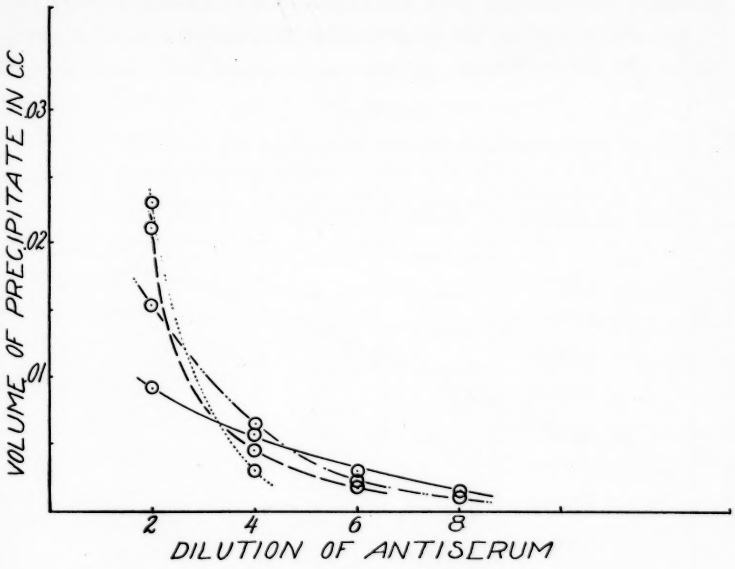

FIG. 9.—Effect of antiserum dilution

From Figure 10 it can be seen that practically a direct relationship exists between the temperature and the volume of precipitate obtained, when working within certain limits. Below 13° C. the results are inconsistent. This is probably due to the influence of the room temperature acting during the time of centrifugation. Above 63° C. it was found difficult to measure the precipitate volumetrically, as the precipitate was very bulky and noticeably colloidal in nature when compared to its granular appearance at lower temperatures.

The reaction was carried out to as high a temperature as 73° C., but the precipitate was too flocculent to pack properly in the thrombocytocrits. With all temperatures above 60° C. a control of normal rabbit serum, diluted as the experimental antiserum, was used in

place of the experimental antiserum. In no case did a precipitate occur in a control, even up to 78° C.

Since a straight line or direct relationship exists in this reaction mixture, it was possible to calculate the rise in volume per degree change in temperature. This was found to be an average of 0.000242 cc. per degree centigrade between the temperatures of 13° C. and 63° C. (see Table IV)

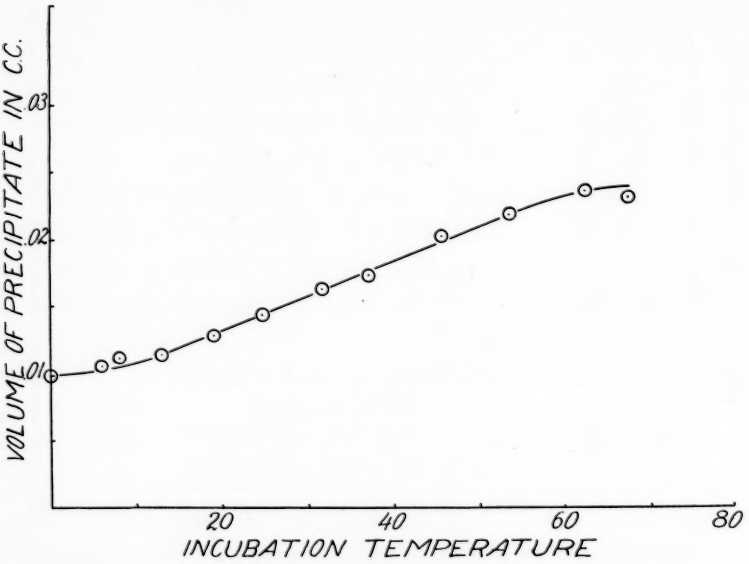

FIG. 10.—Effect of temperature

From the results obtained, it is then possible to ascertain the volumes of precipitate for any temperature within the limits stated.

In any study, also, it is necessary to maintain a constant temperature if the results are to be compared.

Experiment IV. Effect of length of incubation time on precipitate formation.—The conditions of the experiment are: (1) antiserum (rabbit W-80) constant at 0.5 cc. of a 1:5 dilution; (2) antigen (crystalline egg albumin, sample 20) constant at 0.5 cc. of a 1:150 dilution; (3) temperature 37.5° C. ± 2° C; and (4) time of incubation variable.

Figure 11 shows that there is a steady increase in the volume of precipitate obtained after centrifugation for 30 minutes, until the reaction mixture has been incubated from $1\frac{1}{2}$ to 2 hours. When the time of incubation is lengthened beyond this, the measurement of the precipitate is uncertain. The first rise to equilibrium is due to the gradual formation of precipitate, as is seen from the analysis of the supernatant fluid. The antigen was entirely removed from the super-

TABLE IV

EFFECT OF TEMPERATURE ON THE VOLUME OF PRECIPITATE

Temperature ± 1° C.	Mean Value of 30-Minute Reading	Probable Error of Mean	Average Rise per Degree Centigrade
0°	0.00990	0.00033	
			0.00016
6°	0.01069	0.00042	
			0.00019
8°	0.01107	0.00018	
			0.00005
13°	0.01134	0.00012	
			0.00026
19°	0.01295	0.00001	
			0.00024
25°	0.01442	0.00043	
			0.00029
32°	0.01644	0.00015	
			0.00016
37.5°	0.01727	0.00012	
			0.00034
46°	0.02033	0.00013	
			0.00019
54°	0.02187	0.00010	
			0.00021
63°	0.02372	0.00015	
68°	0.02308	0.00022	

natant fluid, as ring tests showed; while precipitins were present in decreasing amounts until at 75 minutes of incubation these were removed entirely. The second apparent rise in volume is then due to an alteration in the nature of the precipitate formed. This was further shown by the fact that the precipitate was difficult to measure volumetrically. The alteration in the nature was, then, one which hindered the packing of the precipitate in the thrombocytocrits, and probably not one of increased precipitate formation. It was inadvisable, for this reason, to study the effect of time any further, as

equilibrium was apparently reached between 1½ and 2 hours of incubation.

The series of readings on the solid graph are those taken after a half hour of centrifugation, so that the period of 30 minutes must necessarily be added to the time of incubation shown to get the total time of the reaction. To the left of the ordinate, however, are indi-

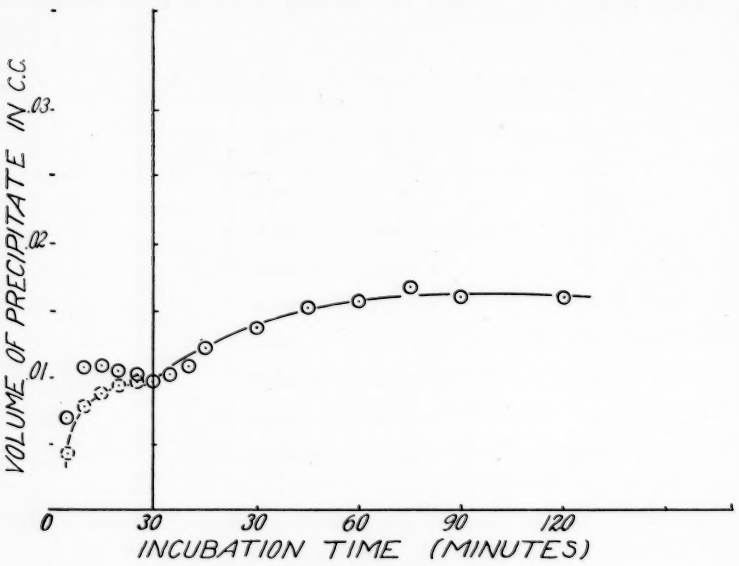

FIG. 11.—Effect of incubation time

cated the readings taken at 5-minute intervals from the time the reaction was set up (the reagents being kept at 37.5° C.). The unconnected points are the actual readings; the connected points (dotted line) are the volumes of precipitate corrected to read as centrifugalized for a 30-minute period, so that they may be compared with the other 30-minute centrifuge readings. To do this, a volume of precipitate was obtained, from a suitable mixture of antigen and antibody kept at 37.5° C. at the start, that would correspond to the volume of precipitate obtained at the 30-minute centrifugation period desired. This column of precipitate was centrifuged for ½ hour

for 5-minute periods, so that from these readings the percentage decrease in volume or the percentage of packing of the precipitate every 5 minutes could be calculated.

These readings from which the packing rate was determined for such a volume of precipitate are shown in Table V. Here it is seen that there is a steady decrease in the percentage of packing of the precipitate for each successive 5-minute period. To make use of this for data obtained in the experiment, however, it is necessary to obtain correction factors for the volumes obtained to make the readings equivalent to half-hour readings instead. Thus, it was found from the

TABLE V

CORRECTION TABLE OF CENTRIFUGE VALUES

Time of Centrifuging (Minutes)	Mean Values	Deviation from 30-Minute Reading	Percentage of Deviation	Readings from Experimental Data	Corrected Readings
5.....	0.01767	0.00702	39.7	0.00704	0.00424
10.....	0.01457	0.00394	27.0	0.01075	0.00785
15.....	0.01308	0.00244	18.6	0.01080	0.0088
20.....	0.01208	0.00144	11.1	0.01058	0.00940
25.....	0.01132	0.00068	6.0	0.01023	0.00961
30.....	0.01064	0.00000	0.0	0.00978	0.00978

table that the 25-minute reading is 6.0 per cent greater than the 30-minute reading; the 20-minute reading is 11.1 per cent greater than the 30-minute reading, etc.; thus, for example, to make the 20-minute reading of the experiment equivalent to the 30-minute reading which would have been obtained had the volume been centrifuged for 10 minutes more, it is but necessary to correct the reading obtained at 20 minutes by 11.1 per cent. The same procedure applies to the other readings obtained. Thus the disconnected curve of the graph was obtained to show the rate of formation of the precipitate during the first half hour of centrifugation.

The nature of the curve obtained is perhaps accounted for when we consider the precipitate formed during this first 30 minutes is removed from solution almost as soon as the particles become of suitable size to be affected by the centrifuge. The precipitate removed from the solution cannot be altered so much as it would be while still suspended.

From the actual readings obtained (those points not connected in the graph), it is seen that the rate of formation of precipitate for the first 10 minutes is much greater than the degree of packing; while between 10 and 15 minutes after the reaction is set up, the rates of formation and packing are equal. After 15 minutes, however, the rate of packing exceeds the rate of formation of precipitate.

Taking the entire curve, then, it can be seen that the rate of reaction is rapid at first until an equilibrium is reached after about $1\frac{1}{2}$ hours of reaction at 37.5° C. It appears that this, then, is the optimal time of precipitate measurement, as a more lengthened incubation period so alters the nature of the precipitate as to make later measurements more undesirable.

Experiment V. Volume of precipitate, as influenced by time and rate of centrifugation.—Constant conditions were employed here of (1) 0.5 cc. of antiserum (rabbit W-80) diluted 1:5, (2) 0.5 cc. of antigen (crystalline egg albumin, sample 20) diluted 1:150, and (3) incubation for 1 hour at a constant temperature of 37.5 °C.

Two centrifuges were used in the experiment. Machine A revolved at the rate of 2,150 r.p.m.; machine B revolved at the rate of 2,250 r.p.m. Readings were made at intervals of 15 minutes as indicated.

From Figure 12 it is seen that there is a rapid decrease in the volume of precipitate at first, but that the rate of packing soon decreases until an equilibrium is reached. The precipitate was not centrifuged to equilibrium, but it can be seen that it would require many hours to obtain constant volume at these speeds.

The rate of centrifugation is also an important factor, especially when constant volume is not to be used as a measure of the reaction. The two curves shown in the graph are similar in nature except for the difference in precipitate volume. This difference is directly due to the greater packing rate as affected by a greater centrifuge speed. As the time of centrifugation is extended, the two curves approach each other, so that in order to have the results similar, when different rates of centrifugation are being employed, one would have to centrifuge for many hours. However, if similar constant-speed controlled centrifuges are used, the results will be rather uniform, and a 30-minute period of centrifugation will suffice.

Experiment VI. Effect of hydrogen ion concentration of formation of precipitate.—Constant quantities of antigen (0.5 cc. of a 1:300 dilution of crystalline egg albumin, sample 21) and antiserum (0.5 cc. of a 1:2 dilution of rabbit 21-W) were used at an incubation temperature of 37.5° C. The pH was varied by using physiological saline diluents of differing hydrogen-ion values. This was accomplished by using a glycine NaOH—HCl buffer series as suggested by Clark.

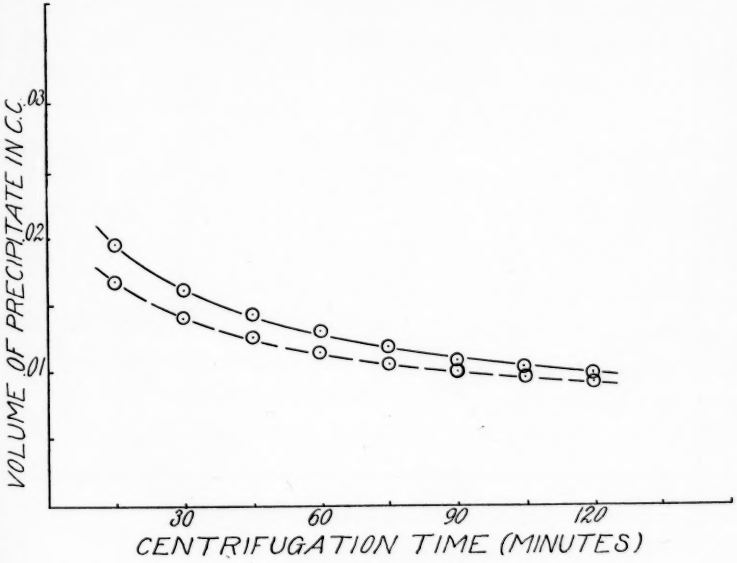

FIG. 12.—Effect of centrifugation time and rate

The pH was determined electrometrically on the individual 1 cc. incubation mixtures, after determining the quantity of precipitate formed, by means of a quin-hydrone electrode. Figure 13 shows the results obtained.

Below pH of 4.6 and above pH of 8.5 the precipitate fails to form, although both antigen and antibody are present in the reaction medium. As the pH of the medium increases, the precipitate is increased in volume very rapidly, until a plateau is reached, which again drops off rapidly in the vicinity of pH of 8. Between pH of 6 and 8 the

quantity of precipitate is approximately constant. It is suggested, then, that when comparable results are desired, a buffered reaction mixture be used to be approximately at the neutral point. It was noticed that the precipitate on the acid side of the range was fine and granular, while on the extreme alkaline side of the curve the precipitate was rather colloidal in nature and slightly more bulky.

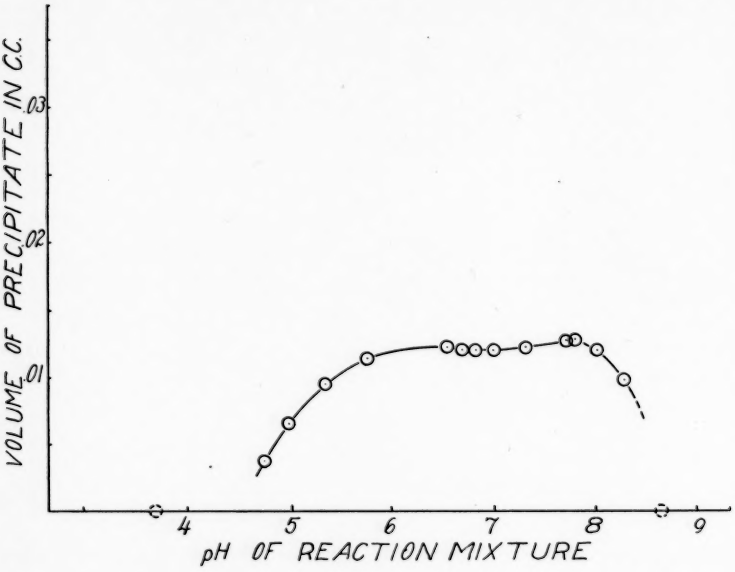

FIG. 13.—Effect of pH

Experiment VII. Effect of salinity of incubation mixture on formation of precipitate.—Two studies were made, one using crystalline egg albumin as antigen, and the other using a serum protein as antigen, reacting in each case with their homologous antiserum. The crystalline egg albumin (sample 20) was diluted 1:300, using 0.5 cc. to react with 0.5 cc. of antiserum (rabbit 21-W) diluted 1:2. Figure 14 shows the results obtained. The dog serum protein antigen (sample 1) was diluted 1:80, using 0.5 cc. to react with 0.5 cc. of antiserum (rabbit 7-W) diluted 1:5. Figure 15 shows the results obtained, which in this case only ran to a saline dilution of 3.3 per cent.

The abscissas in each case show the final concentration of salinity obtained by calculation from the salinity of the original antigen solutions and the diluent employed. The diluent was a series of buffered solutions of varied sodium chloride content, prepared gravimetrically. By this means a final reaction concentration of 27 per cent could be obtained. In order to approach 30 per cent concentration,

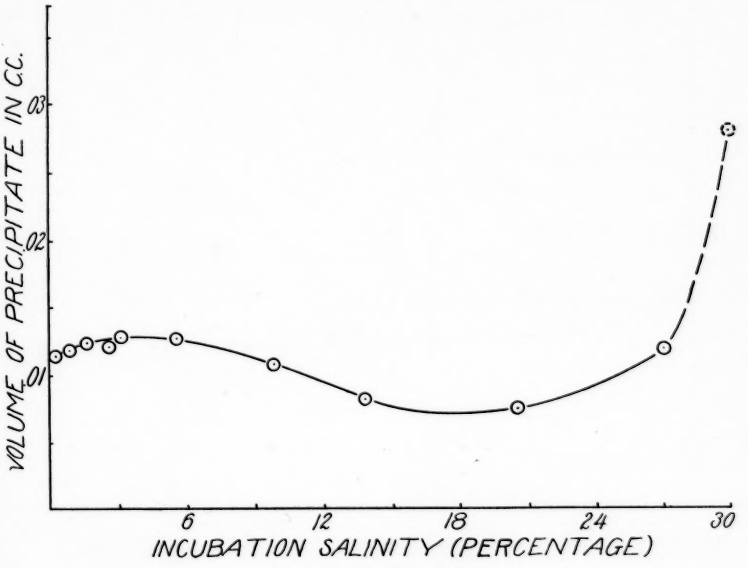

FIG. 14.—Salinity curve

the required weight of solid NaCl was added to each incubation mixture. This probably introduced a slight negligable error, in that the final quantity of antigen and antiserum was slightly greater than 1.0 cc. Controls were run at 27 and 30 per cent saline concentration consisting of the 0.5 cc. dilution of antiserum along with 0.5 cc. of saline instead of the antigen.

From Figure 14 it can be seen that, starting with a saline concentration of 0.29 per cent there is a slow rise to about 3 per cent, when the presence of the saline begins gradually to decrease the quantity of precipitate formed until the low level at about 20 per cent is

reached. From here the curve rises rapidly to the high point at 30 per cent concentration. This last rise is associated with a more flocculent condition of the precipitate and a failure in proper packing of it in the thrombocytocrits. That the last rise shown by the broken line is due to the general salting-out action of the increased saline concentration, and not due to the specific action of antigen on anti-

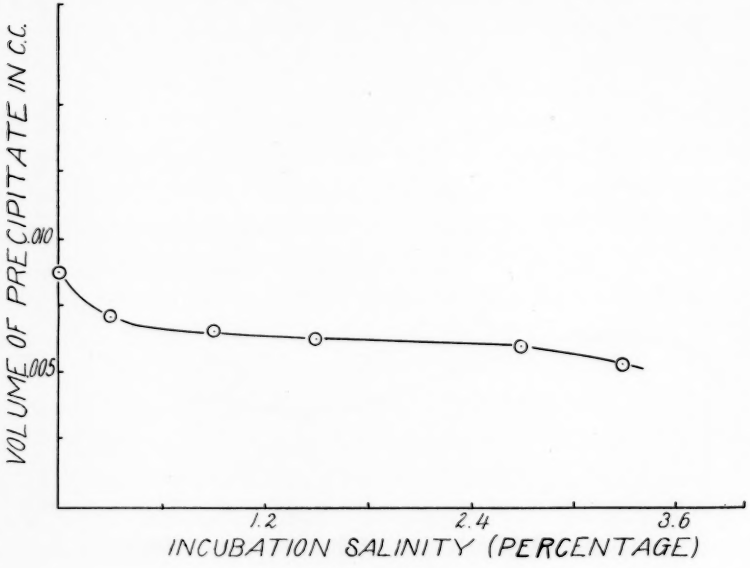

FIG. 15.—Salinity curve

body, was shown by the control tests at 27 per cent and 30 per cent. While a great amount of natural turbidity was observed at the 30 per cent concentration, very little (unmeasurable) was evident at 27 per cent. It is suggested that this general decrease in quantity of precipitate measured up to a salinity of 20 per cent may be due to a general dehydration resulting in a decrease in particle size.

Figure 15 shows the general decrease in precipitate volume with increasing salinity, but the initial rise in volume is absent. This may be due to the fact that serum is a complex of proteins of differing properties and also that an antigen-antiserum ratio was used to pro-

duce but a small amount of precipitate. However, in both graphs it is seen that it is essential to use a constant salinity in serological studies for comparable results.

Experiment VIII. The effect of a foreign protein (serum) on the formation of precipitate from an antigen reacting with its antibody.— This study was suggested from the so-called "conglutination reac-

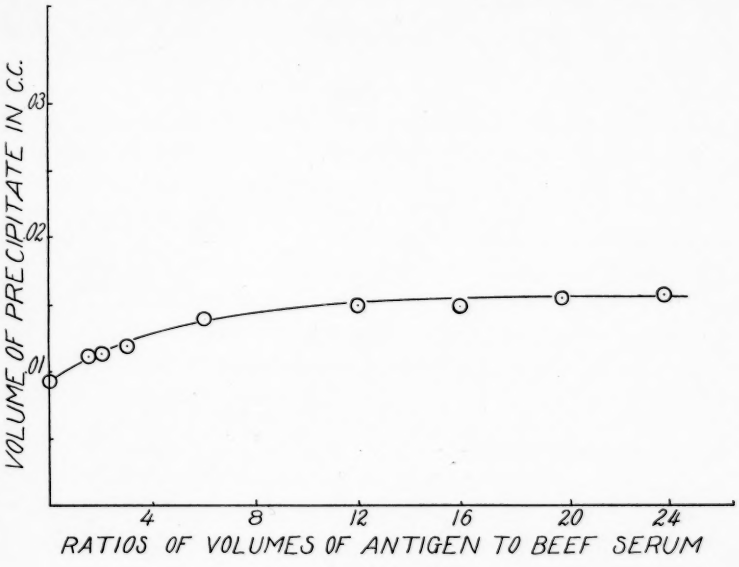

FIG. 16.—Conglutination curve

tion" as employed in bacteriological studies to augment the usual agglutination reaction. However, it need not be considered here whether or not it is a distinct reaction or merely an instance of the general property of adsorption (complement, mid-piece, etc.). It was used in this experiment to determine whether a normal small volume of precipitate could be increased to facilitate satisfactory measurement in the thrombocytocrits.

Figure 16 shows the results obtained from reacting 0.5 cc. antiserum (rabbit R-19) diluted 1:2 with 0.5 cc. of the crystalline egg albumin (sample 22) antigen diluted in normal beef serum of varying

proportions so as to have the final antigen dilution constant at 1:1200.

The quantities of beef serum varied from zero to a ratio of 1 part of antigen diluted in 23 parts of beef serum, or a ratio of 1:24. For example, to make an antigen beef serum ratio of 1:12 with the final antigen content of 1:1,200, an antigen dilution of 1:100 was prepared and 1 part of this mixed with 11 parts of beef serum. As a result the final antigen ratio was 1:1,200, and the ratio of antigen to beef serum in the final antigen dilution was 1:12. This was carried out in this manner for all quantities of beef serum desired in this test, so as to facilitate keeping the antigen constant in dilution for all readings. In reading the abscissa to the right of the ordinate, then, the quantities of beef serum are increasing in a geometric proportion.

From the graph it can be seen that there is an increase in the quantity of precipitate obtained, owing to the addition of a foreign protein. This reaches a plateau with the beef serum present in the proportion of 1 part antigen to 8 parts of beef serum. With further increase in normal beef serum there is no perceptible increase. This augmentation of the normal precipitate volume must be due to an adsorption of the beef serum by the antigen-antibody complex. However, there must be a limit to the adsorbable protein, as evidenced by the failure of a greater amount of beef serum to cause a further increase in volume. If allowances are then made for the nature of the foreign protein, it is obvious that small precipitate volumes can be readily increased to facilitate reading.

DISCUSSION

Since the work of Hamburger in 1905 there seem to have been no careful studies of precipitate volume reported except that of Boyden and Baier in 1929. In his study Hamburger failed to give an account of the range of error of his measurements. There is, however, a paper reported by Mollison in 1924, but the data presented are hardly sufficient to establish the reliability of his technique.

The methods employed in this investigation are superior in that the volumes have been measured by a fairly simple process, the error of which has been definitely stated. Also, by the addition of speed-controlled centrifugation (tachometer) results can be obtained that

will be comparable under all conditions. It is hoped, also, that the use of the probable error, as a check on reliability, will find use among serologists in the future.

The quantitative technique here employed has verified some of the conclusions of earlier workers who used cruder methods. Thus, with regard to the effect of increasing the quantity of antigen in the reaction, it can be seen that the results agree with those formulated by Fleishman and Michaelis (1907). They recognized that the formation of precipitate increases at first with the addition of precipitinogen, then decreases, and with a certain excess of the antigen approaches zero, this being the "zone phenomenon." Zinsser has also noticed the solvent action of excess of antigen, using anti-sheep rabbit serum. Of the recent investigators, Opie (1923) has drawn the same conclusions using crystalline egg albumin (a simple protein antigen).

Dean (1912) described the use of "optimal proportions" of antigen and antibody to produce the zone of most rapid particulation of precipitate as a means of reading the reaction. Dean and Webb (1926) have shown that several zones exist in the precipitin reaction when serum protein antigens are used. In their studies it is shown that the zone of maximal precipitate formation is near the zone of optimal particulation described by Dean (1912). The differences which Dean and Webb describe between the zones of relative antigen and antibody excess are confirmed by the graphs included in this study under Experiment 1. The graphs show a rather continuous relationship between the zones described by Dean and Webb (1926).

The use of the point of optimal proportions as a means of comparison of precipitin curves hardly seems justifiable under the conditions observed from the graphs. First of all, it is a rather difficult process to measure the precipitate at this point with any degree of accuracy, and, secondly, the variations of precipitate volume that is produced by a slight change in the antigen dilution at this part of the curve are very great, and the results may then be very untrustworthy. It is suggested that for comparison of proteins (blood relationships) points be taken on the curve to correspond to dilutions of relative antigen deficiency. It is obvious that a variation in making the antigen dilutions here would alleviate these great errors.

In this study, moreover, a comparison of various proteins has been made. It is apparent that the curves in all cases are approximately alike and show the usual characteristics of the reaction.

With the study of the effect of antiserum dilution it can be seen that there is a decided relationship between the volume of precipitate obtained and the quantity of antiserum present. That this is the correct interpretation can be seen from the graphs where the antigen is present in a high dilution; i.e., it shows no solvent action on the precipitate. When the antiserum is lowered in quantity (higher dilution) so as to remain still in relative excess over the antigen present, a decided drop in precipitate volume occurs. However, if, as in Figure 9, curve 4, the antigen content were nearer the point of relative excess (near the zone of solvent action), then the decrease in antiserum content would throw the antigen in relative excess and this decrease in volume of precipitate would be due to solvent action of excess of antigen. It is noticed from the Figure 9 that this latter solvent action is more pronounced; whereas in the case of Curve 1, showing a relative high antigen dilution, the diminution of precipitate is more gradual. These findings seem to be contrary to those of Cromwell (1925), who, using crystalline egg albumin antigen, states that "all dilutions of the antiserum give a precipitate with the same high dilution of the antigen."

As to the effect of temperature on the reaction, it has long been known that the rate of precipitate formation increases with temperature. For the first time, however, a precise curve (see Figure 10) showing this relation with a simple protein has been obtained. This figure is presented again, along with Figures 11 and 12, to complete the data on the study (Baier, 1930). The practical importance of a study of the relations of temperature is only too obvious. The nature of the precipitins themselves have been studied as influenced by temperature, but it appears of more importance to the writer that the combining or reacting properties of antigen and antibody should be given as influenced by temperature. To be sure, it is likewise important to know the effect of temperature on the specificity and potency of sera when serological studies of taxonomy or the medico-legal aspects are being investigated. The results of the curve as presented show that for comparable readings a definite temperature should be employed; but exact use of 37° C. is surely not required.

The practical importance of a study of the relation of incubation time to the amount of precipitate formed is very great. Obviously, for comparative studies such a time should be used as will just reach the equilibrium point and no more. This is essential, as further incubation apparently causes a change in the nature of the precipitate, rendering measurement difficult and also varying the volume that would normally be obtained. Since the reaction is being used in the quantitative study of animal relationships, readings should be taken not earlier than the 1-hour reading—preferably a 90-minute reading, to allow complete action and thus obtain the most consistent and useful data. Apparently, then, it is not necessary to allow the ingredients to react over a 24-hour period, as is often done in the flocculation test.

The effect of rate of centrifugation is likewise of considerable practical importance. The data secured on Figure 12 includes that from two similar centrifuges with slightly different speeds. From this study it was obvious that for comparable results similar centrifuges revolving at the same speed for the same length of time would have to be used, unless centrifugation to constant volume would be employed. This is obvious from the graph where the volumes of precipitate only begin to approximate each other at the end of $1\frac{1}{2}$ hours. To remove the error of speed of centrifugation, which was observed to vary from day to day, speed-controlled centrifugation is now employed, using a magnetic tachometer, and regulating the speed for all readings at 2,400 r.p.m. by means of a variable resistance.

The effect of the hydrogen-ion concentration is in accord with the results of other investigators in both precipitin and agglutinin studies. Hirsch, using sheep serum and homologous immune serum, found the pH range of precipitins to be between 6 and 9.4. For agglutinins (*Bacillus typhosus* and its immune serum) De Kruif and Northrop found a reaction to occur between pH 3.7 and 9.0, while Mason (1922) showed that a specific precipitate will form between pH 4.5 and 9.5. In the present study, where a simple protein antigen is used, the pH range lies between 4.5 and 8.6, with very little variation in precipitate volume between pH 6 and 8.

The salinity of the reaction medium is also of great importance. Obviously, however, the changes in salinity are only gradual when volumes of precipitate are being read. In general it might be said

that the effect of the increased salt concentration is to result in a decrease in the volume of precipitate obtained, until the salinity reaches such a magnitude to directly affect the serum proteins themselves and thus cause the salting-out effect. The results obtained are in general agreement with the work of Graham-Smith and Sanger (1903), who showed that increase in salinity causes a decrease in precipitate volume up to 7 per cent and that a further increase caused a greater volume of precipitate, but disagrees with the work of Linossier and Lemoine (1902), who found that even 1 per cent of salt impeded precipitation and that 5 per cent completely prevented it. Eisenberg and Volk (1902) reported that 18 per cent of salt has no influence, and Rostoski (1902) showed that 10 per cent saline has very little effect on the reaction, which is in agreement with the present study. It is evident from Figures 14 and 15 that there must be a slight difference in the initial effect of the salinity when crystalline egg albumin and serum are used as antigens. The difference may be due to the complex nature of the protein in the latter case.

Very little need be said about the conglutination curve. It is evident that, with the formation of a specific precipitate from the antigen and the homologous immune serum, a certain amount of nonspecific adsorption of the foreign protein took place. In this manner a previous small amount of precipitate was augmented to facilitate measurement.

SUMMARY AND CONCLUSIONS

1. The quantitative precipitin technique described by Boyden and Baier (1929) has been successfully employed in a quantitative study of some factors affecting the precipitin reaction.

2. The relations of antigen to antibody have been shown quantitatively, by using simple and complex protein antigens, to confirm the results of others (Fleishman and Michaelis, Dean, Dean and Webb, Opie).

3. The volume of precipitate obtained is affected directly according to the quantity of antiserum present in the reaction mixture.

4. The curves obtained using serum, oxyhemoglobin, and crystalline egg albumin as antigens are relatively of the same nature.

5. There is no simple relation between the titer of an antiserum and the quantity of protein injected for antibody production.

6. The volume of precipitate obtained is directly proportional to the reaction temperature under the conditions studied.

7. The volume of precipitate obtained from the mixture of antigen and antibody has reached an equilibrium at the end of 1 hour of incubation at 37.5° C.

8. Using Van Allen thrombocytocrits, the volumes of precipitate obtained, after ½ hour of centrifugation at 2,400 r.p.m., show a fair degree of reliability.

9. The extreme possible error in reading the volumes of precipitate obtained in this study (taken from Table I, picked at random) is ± 10 per cent.

10. The hydrogen ion concentration of the incubation mixture affects the volume of precipitate obtained, with the range of precipitate formation extending from a pH of 4.6 to pH of 8.5. Between pH 6 and 8 there is little change in volume of precipitate.

11. Increase in the salinity of the incubation mixture of antigen and antibody causes a progressive slight decrease in volume of precipitate up to 20 per cent salinity. From 20 to 30 per cent salinity the salting-out effect of the serum proteins is evident.

12. The presence of a foreign protein (beef serum) in the reaction mixture of antigen and antibody causes an increase in the volume of precipitate obtained.

13. The probable error has been used in this study as a measure of the degree of reliability to be placed on the results.

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